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Bacteriological studies
on deep areas of
carious dentine

BY STIG EDWARDSSON

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Bacteriological studies on deep areas of carious dentine

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Bacteriological studies
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To Ulla

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1. Introduction

HISTORICAL

In 1881 Underwood and Millis reported "micrococci, oval and rod-shaped bacteria" in sections of decayed dentine. They also showed that the bacterial population decreased towards the deeper layers of the carious lesion. They concluded: "We consider that caries is absolutely dependent upon the presence and proliferation of organisms." Many investigations have since been performed to elucidate the bacterial flora of deep dentine caries and the possible effect of these microorganisms on intact or decalcified dentine.

In early studies many investigators have found preponderantly streptococci and lactobacilli in carious dentine (for a review see Cox 1952). Knighton (1939) observed *Candida* and Matthews *et al.* (1949) staphylococci. *Leptotrix innominata* of Miller were cultured by Goadby (1903) and Jay (1927).

Gram negative microorganisms have also been observed. Jay (1927) reported the presence of Gram negative bacilli which later on was suggested to be *Bacteroides spp.* (Macdonald 1962). Bibby and Hine (1938) found abundant of Gram negative bacilli and fusiforms in direct smears of carious dentine.

Some reports deal with spore forming bacteria possibly occurring in dentine caries (Goadby 1903, Kligler 1915). Hartles and McDonald (1951) who studied the growth of anaerobic organisms from carious teeth, isolated *Clostridium welchii* from approximately 60 per cent of 252 carious teeth but from only 10 per cent of 48 apparently sound teeth. The isolated organisms produced an enzyme which was able to break down decalcified dentine. Hartles and McDonald concluded that "the possibility arises that *Clostridium welchii* may be important in the carious process". During the same period Engel (1950) showed that filtrate of *Clostridium welchii*, which contain collagenase, could not release nitrogen from undecalcified dentine but liberated nitrogen from decalcified dentine in proportion to the extent of the decalcification.

After enucleating the decalcified dentine and exposure of the junction

between decalcified and intact dentine, Burnett and Scherp (1951 a, b, 1952) collected material from the junction with an excavator. They incubated the material anaerobically and aerobically on different agar media. Only three groups of microorganisms were regularly isolated viz. Gram positive cocci, lactobacilli and *Actinomyces*. Over 90 per cent of the counts were made up of cocci resembling enterococci and micrococci. The *Actinomyces*-like organisms regularly constituted about 5 per cent of the count; they were acidogenic and acted on intact dentine with the formation of a dark brown pigment resembling that often seen in natural caries. Material was also collected from more superficial levels of the lesions. Relatively abundant amounts of proteolytic bacteria were found at these levels. Although heterogenous, they could possibly be assigned to genera such as *Streptococcus*, *Staphylococcus*, *Bacillus* and *Pseudomonas*. Some of these proteolytic bacteria lysed decalcified dentine.

In 1954 and 1958 Onisi and Nuckolls reported an investigation of the bacterial flora recovered from pigmented carious lesions of the dentine. Realizing the importance of control of contamination by microorganisms from the tooth surface when specimens are taken from the deepest part of the dentine lesion, these investigators published a detailed report concerning the contamination problems. They disinfected the surfaces by heating the teeth in paraffin at $+200^{\circ}\text{C}$ to kill the organisms on the external surface of the teeth, while the biota in the internal part of the lesions remained viable. With an aseptic technique, the teeth were then split through the carious lesion and by drilling, samples were collected from the border area of the lesion. This area was regarded as the innermost part of the affected dentine that had been exposed by the splitting. Although hard the area showed definite discoloration. The specimens were cultured on various sorts of agar media and incubated under micro-aerophilic conditions. Pleomorphic Gram positive microorganisms dominated and mostly resembled *Actinomyces* and *Nocardia*. These bacteria were isolated "from the samples" in about 38 per cent. Isolates identified as *Corynebacterium* were observed in 16 per cent and pleomorphic streptococci in about 19 per cent. The remaining isolates consisted of Gram positive cocci and rods presumably belonging to the genera *Staphylococcus*, *Gaffkya* and *Bacillus*. No Gram negative bacteria were observed. Onisi and Nuckolls concluded that the large number of isolates resembling *Actinomyces* and *Nocardia* may indicate a possible relationship between these organisms and caries in dentine.

In 1957 Roth studied the bacterial flora of the dentine lesion with special reference to organisms capable of lysing hide powder. She found that a pleomorphic often coccoid, aerobic to facultatively anaerobic rod

was the organism most regularly present. Roth identified these isolates as species of *Nocardia* and proposed the name *Nocardia dentocariosus*. This species has recently been considered a new genus *Rothia denticariosus* in the family *Actinomycetaceae* (Georg and Brown 1967). Other organisms which Roth frequently observed were *Gaffkya* and micrococci, while anaerobic streptococci and *Actinomyces*-like bacteria were found difficult to culture and thus not studied to the same extent as the other isolates. These five groups of organisms were demonstrated in all areas of the carious dentine, many of them being capable of breaking down a denatured collagen incorporated in the bacteriological medium used. On the basis of these observations Roth postulated that the presence of these five groups of more or less proteolytic bacteria in the carious lesion suggests that they play a role in the proteolysis of collagen, an organic constituent of teeth in the penetration of the carious process.

A new species of *Actinomyces* was described by Batty (1958). This microorganism differed from previously described species of *Actinomyces* in its greater tolerance to oxygen and in producing a red pigment. Batty proposed the name *Actinomyces odontolyticus* for the organism. This name has since been accepted as a species in the genus *Actinomyces* (Pine 1970). Batty found that *Actinomyces odontolyticus* was regularly demonstrable in the deeper part of the carious dentine, but not in early enamel caries and only rarely in early dentine caries. The proportion of the total bacterial growth represented by *Actinomyces odontolyticus* varied considerably between the samples of carious material analysed. Batty concluded that, whether or not *Actinomyces odontolyticus* is the cause or one of the causes of dentine caries can not be decided in the absence of some feasible methods of producing experimental caries under controlled conditions.

Yardeni *et al.* (1959) studied the bacterial flora within the caries lesion in an endeavour to elucidate bacterial invasion by acceptable bacteriologic criteria and to isolate the primary invaders and find out whether they belong to a well defined group. The study was undertaken on teeth to be restored. The organisms most often isolated were identified as streptococci. They appeared as pure cultures in about 75 per cent of the teeth analysed. Biochemical and serological typing of the strains showed that the majority of them belonged to the viridans group. Some of the isolates resembled *Streptococcus sanguis* and *Streptococcus faecalis*. Most of the pure cultures obtained were found in samples taken from teeth with superficial enamel caries where discoloration was the only macroscopic sign of an initial attack. Lactobacilli were isolated in pure culture from only one of the teeth analysed. In deeper cavities mixed

cultures of micrococci, diptheroids, lactobacilli and filamentous organisms were often observed.

Canby and Burnett (1960) published a short communication about a study of the microorganisms of the deep dentine caries. To minimize extraneous contamination, samples were obtained only from carious lesions of extracted teeth containing a reasonable amount of decalcified but leathery dentine. Attempts were made to obtain a culture sample not contaminated with diverse flora of superficial portions of the lesion. The organisms isolated were uniformly Gram positive non-motile, non-sporulating, aciduric and acidogenic bacilli resembling those of the genus *Lactobacillus*.

Camilleri and Bowen (1963) isolated and classified lactobacilli from subsurface carious dentine. The material used was excavated from the depth of the lesion. Homofermentative lactobacilli outnumbered the heterofermentative variety by 3.5 : 1.0. Of the population, *Lactobacillus casei* was the most frequent isolate (63 per cent) followed by *Lactobacillus fermenti* (14 per cent). The remaining strains isolated were *Lactobacillus plantarum*, *Lactobacillus salivarius* and *Lactobacillus buchneri*. In the 7 teeth examined, *Lactobacillus casei* was demonstrated in 6 and *Lactobacillus fermenti* in 5. Two or more different strains were isolated from 5 teeth.

Using bacteriological and histological methods, Lichtenberg-Crone (1963, 1968) studied the dentine cavity to assess the effectiveness of excavation as a means of removing microorganisms and to study the bacterial flora of the deepest layer of carious dentine. Scrapings from the hard dentine wall in the cavity were cultured in semisolid broth. After excavation the teeth were decalcified, sectioned and Gram stained. Twenty six per cent of the teeth had given growth on culture whereas the histological examination demonstrated microorganisms in the dentine tubules of 52 per cent. The discrepancy was explained by the presence of a large number of non-viable bacterial cells in the dentine. The viable microorganisms recovered from the innermost zone of the lesion were preponderantly streptococci, staphylococci and Gram positive rods. These bacteria were acidogenic and to a certain extent aciduric. No proteolytic activity of microorganisms in deep dentine caries was detected.

Plathner and Binus (1965) examined the aerobic and facultative anaerobic bacterial flora of deep carious dentine layers. Streptococci mostly of the viridans group, dominated. In 2 per cent of the cases studied, the authors observed microorganisms identified as streptococci belonging to Lancefield's serological group D.

On the basis of the hypothesis that streptococci are closely related to deep dentine caries, Parikh *et al.* (1965) studied the presence of these

organisms in the cavity with special reference to their possible ability to produce hyaluronidase. Eighty extracted human teeth were studied and streptococci were isolated from 50 of them. In 45 cases streptococci were found together with other microorganisms such as lactobacilli, staphylococci, *Neisseria*-like cocci, Gram negative rods or yeasts. In 5 cases streptococci were found in pure cultures and in 8 cases lactobacilli were the only bacteria observed. Hyaluronidase-producing streptococci were isolated from 14 of the total number of specimens cultivated. Using histochemical methods Toto (1967) found that streptococcal invasion of the dentine tubules is accompanied by a loss of the acid and neutral mucopolysaccharides within the tubules. Later Toto *et al.* (1968) observed that streptococci isolated from carious dentine could derive their carbon solely from a medium composed of a salt solution with added chondroitin sulphate or hyaluronic acid.

Toto and Prendergast (1968) confirmed the above-mentioned findings. They treated material from the dentine carious lesion with human anti-streptococcal hyaluronidase anti-serum and showed that the organisms in the dentine tubules of specimens from carious lesions reacted to hyaluronidase anti-serum in all samples except in those from the controls. They concluded that their findings supported the view that the destructive process of dentine in carious teeth, which is characterized by loss of acid and neutral mucopolysaccharides, is mediated by hyaluronidase produced by microorganisms, mainly streptococci, invading the dentine.

Recently Shovlin and Gillis (1969) isolated bacteria from "the base of the dentine lesion for the purpose to examine their biochemical and antigenic properties". The samples were isolated from teeth undergoing treatment. Lactobacilli were isolated from 22 of the 25 teeth examined. In 8 instances they were the only organisms found. In 7 teeth lactobacilli were found together with other Gram positive rods, in 3 cases together with streptococci and in another 3 with yeasts. One of the teeth harboured staphylococci and lactobacilli. When discussing their results, they felt that isolation of only lactobacilli in 8 of the teeth may indicate that this was the only organism present in the extreme depth of the lesion. But they also offered alternative explanations. They thought that other organisms may be present but had not been demonstrated by the bacteriological method used. Slowly growing filamentous organisms, as reported by other investigators, may have been overgrown in the broth medium used at the primary cultivation of the specimens.

McKay (1969, 1971 personal communication) confirmed Shovlin's and Gilles' (1969) observations in a histological and bacteriological investigation of deep dentine lesions. According to McKay only orga-

nisms resembling *Lactobacillaceae* are present in the "primary wave" of bacteria in dentine caries.

Kreter and Dornauf (1970) studied the aerobic and facultatively anaerobic flora of carious dentine with special reference to the polysaccharide-producing streptococci. They investigated specimens from 480 teeth and found no flora typical of the lesion but polysaccharide-producing streptococci resembling *Streptococcus sanguis* and *Streptococcus mutans* were more regularly found in samples from "initial caries lesion" than in the deep dentine.

COMMENTS

In the literature concerned with the microflora of the dentine caries lesion the opinion generally is that Gram positive bacteria are common in the deepest part of the region. Yet it is not known with certainty whether any particular genus or family of bacteria dominates the microbiota. Neither is it possible to assume the existence of a typical mixed flora composed of certain genera or families. Furthermore, in the last decade, much interest has been focused on the extracellular polysaccharide-producing microorganisms *Streptococcus sanguis* and *Streptococcus mutans*. Many of these studies concern the relationship between these bacteria in plaque and the development of enamel carious lesions (for review see Keyes 1968, Gibbons 1968, de Stoppelaar 1971, Scherp 1971). Few reports are available on the presence of *Streptococcus sanguis* and *Streptococcus mutans* in the deep dentine caries lesion (Clarke 1924, Maclean 1927, Yardeni *et al.* 1959, Kreter and Dornauf 1970). Only little is known about the amount of these streptococci in relation to the total microbiota of dentine caries.

The "pioneer" bacteria close to the advancing front of dentine caries should be seriously considered as etiologically significant. The reactions which these organisms are able to effect would be indicative of the various processes which occur in the initial stages of the disease in dentine (Burnett and Scherp 1951 b). But, in this partly decalcified region, the number of cultivable organisms is much smaller than in the fully developed lesion (Burnett and Scherp 1951 a). This fact and because of other reasons—which will be discussed further—it may be difficult to collect bacteriological samples from this deep area.

First, disinfection of the surfaces of extracted teeth, before sampling from the lesion, should not be such as to influence the microflora in the deep part of the dentine cavity.

Several methods have been used to disinfect the tooth surface before

sampling, McIntosh *et al.* (1924) flamed the extracted teeth, Clarke (1924) and Maclean (1927) dipped the freshly extracted teeth momentarily into boiling water. Tunnicliff and Hammond (1938) scrubbed the teeth with soap and water. Andersson and Rettger (1937) cleaned the outer surface of the extracted teeth with diluted alcohol. Lichtenberg-Crone (1963, 1968) rinsed the teeth thoroughly under running tap water. Working with *in vivo* material, Roth (1957), Yardeni *et al.* (1959) and Shovlin and Gillis (1969) isolated the teeth with rubberdam followed by washing the tooth or the floor of the cavity with alcohol or alcohol and merthiolate. However, the effectiveness of the methods used have many times not been properly tested. A search of the literature revealed only one investigation (Onisi and Nuckolls 1954) dealing with the problem in detail. After a thorough study, Onisi and Nuckolls found that the tooth surface could be adequately disinfected by heating the tooth in paraffin at 200°C for 1.0—2.5 seconds.

Second, the sampling technique should be such as to permit isolations of the biota of the deep part of the lesion without contamination by secondary invaders from the lesion or from the atmosphere.

A common method used is to enucleate the decalcified dentine from the outside of the tooth and then collect the sample by scraping the junction between decalcified and intact dentine (Burnett and Scherp 1951 b, Roth 1957, Yardeni *et al.* 1959, Lichtenberg-Crone 1968, Toto *et al.* 1968, Shovlin and Gillis 1969 and others). In other investigations the teeth have been split at the centre of the lesion, so as to expose the inner part of the area affected. The samples are then taken from the border of the lesion (Clarke 1924, Maclean 1927, Onisi and Nuckolls 1954, Davies and Nemes 1955, Parikh *et al.* 1965). The possibility of observing the borderline when taking the samples in the latter method is advantageous. But, both methods may give rise to bacterial contamination. In the former method, as pointed out by Dorfman *et al.* (1943), organisms are apt to be carried from the superficial to the deep part of the dentine. In the latter method, bacterial contamination may occur in association with the splitting of the tooth owing to dentine fluid flow or capillary force from the fully developed lesion into the sampling area. This may change the proportion between the bacteria in the samples taken and thereby give misleading results.

Clinically intact dentine beneath the carious lesion is reported not to contain any microorganism immediately adjunct to the lesion (Miller 1891, Stephan *et al.* 1943, MacGregor *et al.* 1956, Fusayama *et al.* 1966). When studying the bacterial flora of deep dentine caries, it therefore seems appropriate to start in the intact dentine of the pulpal wall and

work towards the lesion. This approach has been used by only few investigators and only to ascertain whether cultivable microorganisms occur in the border area of sound dentine (Dorfman *et al.* 1943, Jolly and Sullivan 1960). Neither Dorfman *et al.* nor Jolly and Sullivan were able to demonstrate any cultivable microorganisms in sound dentine but regularly found growth of bacteria in samples of decalcified soft lesions.

Not only the microflora of the fully developed carious lesion, but also airborne secondary invaders can contaminate the samples and disturb the results. Such contamination may occur during preparation as well as during inoculation of the substrates. Little, if any, interest has been focused on this source of error in studies of the microflora of the dentine. There is evidence that the microflora of the human skin is the principal source of the airborne bacteria (Bourdillon *et al.* 1948, Blowers *et al.* 1955, Bernard *et al.* 1965). The major part of this skin microflora is composed of Gram positive bacteria (Kligman 1965) including anaerobic species of *Corynebacterium* (*Propionibacterium*) *acnes* which are considered as the most common culture contaminant when working with anaerobic bacteria (Cato *et al.* 1970).

Third, the culture media should be such as to meet the requirements of bacteria with short and long generation times.

In several investigations primary culture of dentine material has been performed only in broth or in semisolid broth (Canby and Bernier 1936, Dorfman *et al.* 1943, Davies and Nemes 1955, Jolly and Sullivan 1960, Whitehead *et al.* 1960, Lichtenberg-Crone 1963 and 1968, Shovlin and Gillis 1969). In other studies interest has been focused only on certain families or genera of bacteria by using selective media for the primary cultivation (Andersson and Rettger 1937, Roth 1957 and others).

A complex fluid or semifluid medium containing both basic and accessory growth factors usually gives growth more often than agar media (McClung and Lindberg 1957, Willis 1960, Möller 1966). But broth medium may favour organisms with a short generation time i.e. streptococci which will thus overgrow other organisms with long generation times. Yardeni *et al.* (1959) found only streptococci and micrococci when dentine material from the caries lesion was cultured in broth medium, but also rod-like organisms when the specimens were cultured on agar medium. Shovlin and Gillis (1969) used broth when culturing material from deep dentine caries. They could not demonstrate filamentous organisms although such microorganisms have been reported by other investigators. When discussing their results they suggested that the slowly growing filamentous organisms may have been overgrown in the broth culture. If a suitable solid medium is used and the number

of microorganisms growing on the surface are not too numerous, the medium will, as a rule, support growth of all types of bacteria irrespective of the generation time.

Thus, when studying the microflora of the dentine lesion, both broth and agar media should be used.

Fourth, the oxygen sensitivity of various anaerobic bacteria should be taken into account. Anaerobic conditions characterize the dentine carious lesion (Loesche and Syed 1973). Incubation methods creating a low oxygen tension should be considered in order to obtain optimal growth conditions.

When culturing material from dentine carious lesions, Burnett and Scherp (1951 a) demonstrated an anaerobic to aerobic recovery ratio of about 2 to 1, while Loesche and Syed (1973), in a study of the same area with a more developed technique, found a ratio of 7 to 1. Onisi and Nuckolls (1954) incubated their specimens under microaerophilic conditions in candle jars. They found that certain microorganisms grew very slowly under these conditions, but more rapidly in an anaerobic atmosphere. The specimens therefore were incubated for 7 days under microaerophilic conditions followed by four days anaerobic incubation (phosphorus jar). For anaerobic bacteria the initiation of the growth from the inoculation is the most critical phase (Holme 1967, Aranki *et al.* 1969). To prevent the inoculum from dying, anaerobic incubation should be started immediately (McClung and Lindberg 1957, Willis 1960, Möller 1966, Cato *et al.* 1970, Aranki *et al.* 1969).

Furthermore, it has been observed that, also growth of facultative anaerobic oral and pharyngeal bacteria is promoted by an anaerobic milieu (Gilder and Granich 1947, Howell *et al.* 1959, Pine *et al.* 1960). The requirement of certain growth factors also seems to be less pronounced in an anaerobic than in an aerobic milieu (Gilder and Granich 1947, Möller 1966, Carlsson 1970 a, b and 1971).

The incubation atmosphere should have not only a low oxygen tension but a CO₂-tension above that existing in the normal atmosphere as it has been shown that several oral microorganisms such as strains belonging to *Actinomycetaceae* (Pine and Howell 1956, Howell *et al.* 1959, Pine *et al.* 1960, Howell and Jordan 1963), and various streptococci (Carlsson 1970 a, b and 1971) thrive better if the CO₂-content of the incubation milieu is increased above that existing in the normal atmosphere.

Fifth, the minimal incubation time for different microorganisms varies considerably, not only depending on the generation time but also on the number and condition of the cells present (Möller 1966). Many strains

belonging to genus *Actinomyces* are known to require long incubation at least of primary cultures (Rosebury *et al.* 1944, Howell *et al.* 1962). This is also true when nutritionally rich media are used (Möller 1966). When studying specimens from infected root canals Möller observed that although in about 95 per cent of the positive samples obtained, growth was demonstrated on the 4th—5th day it was sometimes necessary to prolong the incubation time to 15 days before growth appeared. It is therefore important to use incubation times meeting the requirements of both rapid and slow growing microorganisms.

Summarizing, in studies of the bacterial flora of the deep dentine caries important factors capable of influencing the results are (1) the disinfection procedures of the tooth surface, (2) the method used for collecting the material and (3) the bacteriological techniques. In previous studies in the field these methodological problems have received but little attention. Onisi and Nuckolls (1954, 1958) have reviewed the reports up to 1953. They are of the opinion that few, if any, of the studies undertaken are based on representative samples. Even in recent publications methodological problems have received little attention.

Aim of the study

In recent years considerable interest has been focused on those bacteria which are assumed to be involved in plaque formation and in enamel caries (for reviews, see Scherp 1971, de Stoppelaar 1971, Gibbons and van Houte 1973). Except for the recent study on the bacterial flora of both plaque and underlying soft carious dentine by Loesche and Syed (1973) and the studies by Sumney and Jordan (1973, 1974) on the bacteria in human root surface caries lesions less attention has been given to those specific bacteria, if any, which might be involved in the pathological process in carious dentine.

A search of the literature revealed little information on the incidence of plaque forming and caries inducing bacteria in the advancing front of carious lesions and their occurrence in proportion to other bacteria present in the area. Moreover, previous studies on the bacterial flora of carious dentine have shown that the biota consists partly of organisms which have only been described as "Gram positive pleomorphic rods" or "Gram positive filaments". Further knowledge about the identity of the flora in the advancing bacterial front line seems therefore of interest to achieve. Such knowledge could have a special value, since it has been postulated that "microorganisms regularly present in large numbers in close proximity to the advancing front of the dentine carious lesion may be considered as etiologically significant (Burnett and Scherp 1951 b).

The aims of the present investigation therefore were:

1. to isolate the cultivable bacterial flora of the advancing front of the carious dentine lesion.
2. to identify isolated bacteria and to assess the relative proportions of different bacteriological families and genera.

2. Collection of material and preliminary identification

A study of the bacterial flora of deep dentine carious lesions involves specific methodological problems. Some of these have been discussed in Chapter 1. As far as possible these problems were considered when the technique for the present study was worked out. In this chapter the technique for disinfection of the tooth surfaces before the sampling of material is described and connected pilot studies are reported. Sampling technique as well as choice of culture media and incubation time are presented. Bacteriological investigations performed to check the preparation of culture media are described. A report is given of the results of the gradually improved technique for reducing bacteriological contamination during the collection of the specimens from the teeth and during the preparation of the culture media.

After isolating but before lyophilizing the strains, only a preliminary description of each isolate was possible. This description was made in order to check on the purity of the lyophilized culture after reconstitution for further characterization. This chapter also reports on the preliminary description of the isolates.

MATERIAL AND METHODS

Disinfection of the tooth surface before sampling

A solution of iodine, ether and colofonium has been used by Kelstrup (1966) as a varnish to avoid contamination from the tooth surface and gingival margin when samples were taken from the gingival sulcus. The disinfecting action of this varnish on the bacterial flora of extracted teeth was tested.

Forty-six teeth were selected for the study. Each tooth had at least one clinically intact surface and one carious surface where the lesion had penetrated into the dentine. Immediately after extraction each tooth was placed in VMG I solution (Möller 1966) and transported to the laboratory. Within one hour the tooth was rinsed in sterile distilled

water and dried with sterile gauze. The tooth was immediately dipped twice in the iodine varnish, each time for about one second. It was stored at $+6^{\circ}\text{C}$ in a sterile petri dish for 15 minutes until the varnish was dry. After mechanical removal of the varnish under a stereomicroscope ($\times 25$ magnification), the surface was washed with sterile 5 per cent (w/v) sodium thiosulfate solution (Möller 1966) and distilled water to eliminate residues of the varnish.

The following surfaces were tested, (I) clinically intact surfaces of enamel, (II) surfaces with carious lesions and (III) after enucleating parts of the soft dentine, samples were taken from the deeper part of the lesions. Small cotton swabs were moistened with Brain-Heart Infusion broth (Difco 0037-01) and rubbed over the surface. Care was taken to avoid contact between the swab and the remaining iodine varnish in the periphery of the test surfaces, as such a contact may seriously influence the results of the sampling (Möller 1966). The cotton swabs were inoculated in Brain-Heart Infusion broth and incubated at $+37^{\circ}\text{C}$ for 3 weeks. Bacterial growth was registered and checked with Gram stained smears.

Material for the main study

Eighty-seven permanent teeth with occlusal or smooth surface carious lesions extending into the dentine but not to the pulp were collected. Most of the teeth were 3rd permanent molars since other teeth with such lesions were usually not extracted. In the part of the dentition from which the tooth was extracted, the caries activity was estimated clinically.

Immediately after extraction the tooth was rinsed in sterile saline to eliminate blood. Remaining soft tissue was removed mechanically. The tooth was transferred to a bottle containing 12 ml of VMG I solution and transported to the laboratory, where the bottle was placed in a refrigerator ($+6^{\circ}\text{C}$). The time between the extraction of the tooth and the sampling from the lesion never exceeded four hours.

Sampling technique

The root of the tooth was sawn off at the enamel-cemental junction and the pulp tissue was removed. With a sterile drill and under moisture of VMG I solution, the cavity of the pulp was widened in those cases where it was narrow and difficult to inspect. The dentine wall under the carious lesion was examined with a stereomicroscope (magnification $\times 16$ —40). If the wall was discoloured or softened the tooth was discarded. Immediately after this inspection the tooth was dipped in

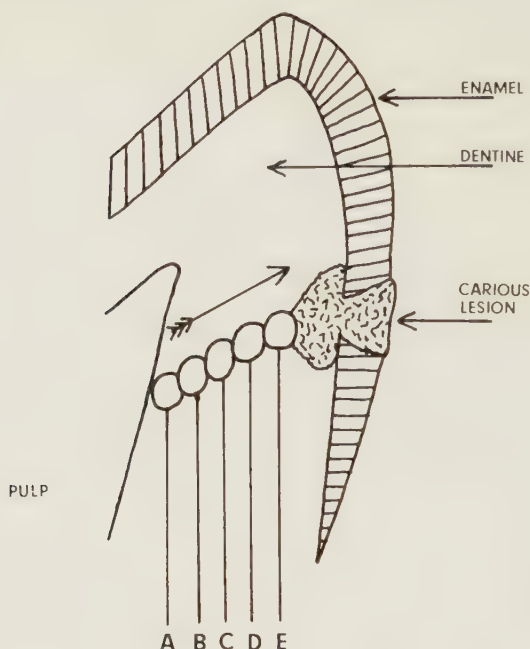


Fig. 2: 1. Dentine has been removed one portion at a time. The first portion (A in figure) always consisted of clinically intact dentine from the pulpal wall. The last portion (E in figure) consisted of material from the soft necrotic carious tissue. As a rule 4—5 portions were taken out from each tooth. A maximum of eight portions were obtained from certain teeth.

the iodine varnish, placed in a sterile petri dish in a refrigerator for 15 minutes to allow the varnish to harden. During the following work an aseptic technique was used (see pages 23—24 and Table 2: 7). Dentine samples were collected using a stereomicroscope. The iodine varnish on the pulpal dentine wall over the lesion was removed mechanically and the surface was rinsed with VMG I solution. With a handpiece and drills (Rotadent no. 1, Dentalborrfabriken AB, Malmö, Sweden) dentine was removed one portion at a time approaching the carious lesion as shown in figure 2: 1. The last portion was taken from the necrotic soft dentine. The work was performed under humidification with VMG I solution. To avoid overheating, grinding was performed at low speed

(about 150 r.p.m.). The rotation speed was controlled with a rheostat (Ultrac AB, Gothenburg, Sweden).

Each portion of material was homogenized in a drop of VMG I solution in a glass mortar (depth and diameter were approximately 3 mm) followed by immediate inoculation on bacteriological substrates. These inoculation procedures are described below.

Sample sizes

In a separate series of 6 teeth, the dry weight of 6 portions per tooth were calculated. After homogenization each whole portion was weighed by the method of Glantz (1969) and Glantz and Svensson (1970). The dry-weight of the test pieces was determined to the nearest 0.01 mg.

In order to calculate the size of the dentine chips in the portions, which were inoculated on the substrates, the dentine particles were measured in a microscope with an eyepiece micrometer after grinding in the mortar. Samples from three separate freshly extracted clinically intact teeth were collected and homogenized by the method described above. In all 8 dentine portions from 3 different teeth were taken and the size of chips observed in 16 squares (1/250 c mm, depth 0.1 mm) of slide counting chamber was calculated.

Culture media and incubation procedures

After homogenization the material was mixed with 0.7 ml VMG I solution. The inoculum on the blood agar plates (Brain-Liver-Heart, Difco 0058-01, supplemented with 1.5 per cent agar (w/v) and 5 per cent (v/v) human bank blood) consisted of three drops of this mixture from a 1 ml pipette or a syringe. The blood agar plates were used as soon as possible after their preparation—mostly the next day. The rest of the material in VMG I solution was inoculated into one screw-capped test tube containing 10 ml TA-Ser medium (Möller 1966).

The substrates were incubated as follows:

	Incubation	Subsequently called
One blood agar plate	7 days in 94 % H ₂ + 6 % CO ₂	7 H
One blood agar plate	2—3 days in 94 % H ₂ + 6 % CO ₂	2 H
One blood agar plate	2—3 days in air	2 A
Ta-Ser medium	14 days in air	T

An anaerobic cultivation atmosphere was achieved by the hydrogen combustion method and the oxygen-free condition was tested with the MGAP-indicator (Möller 1966). Among the anaerobic bacteria, there exists different degrees of sensitivity to molecular oxygen (Loesche 1969). To meet the requirements of sensitive bacteria, the sampling procedure should involve a minimal contact with oxygen. Therefore, the following arrangement was used. The blood agar medium was used as fresh as possible—usually the day after the preparation. The TA-Ser medium was freshly prepared. During the whole sampling procedures, the sampling area was humidified with VMG I solution which has a viability-preserving capacity for bacterial cells (Möller 1966). VMG I solution was also used for homogenizing and diluting the material. To compensate for the prolonged lag phase that anaerobic micro-organisms may have when inoculated at high Eh into an otherwise satisfactory medium (Smith and Holdeman 1968), one of the blood agar plates was incubated anaerobically for 7 days before examination and the TA-Ser medium 14 days before being discharged as negative.

The blood agar plates were analysed under the stereomicroscope. Colony forming units (CFU) with different morphology were isolated. If a sufficient number of CFU were present, 5—10 of each morphological type were Gram stained in order to check the correlation between the cell morphology and the colony forms. The Gram stained smears were prepared according to Preston and Morell (1962). During a later part of the study staining was also performed according to the method for studying anaerobic bacteria (McClung and Lindberg 1957).

Two or three CFU of each type were isolated for further study. If the CFU were of small size, they were inoculated in TA-Ser medium. CFU of sufficient size were inoculated on blood agar. The cell morphology was checked again both with smears from TA-Ser medium and from blood agar. In order to check that pure cultures were obtained, the isolates were—via TA-Ser medium—recultured on blood agar. These procedures were made at least once. When colonies of constant appearance were present, as observed in stereomicroscope, the culture was considered as pure. The isolates were maintained both on blood agar and in TA-Ser medium at $+6^{\circ}\text{C}$ and subcultured every second week. As soon as possible each isolate was lyophilized and stored at -18°C .

The following pre-requisites served as a guide for the selection of teeth for the further studies: (1) The sample from the pulpal wall (A-portion) should not contain any cultivable bacteria that might be derived from the dentine. (2) The first portion with visible bacterial growth, following portion A, was selected for analysis on the assumption that (3) the subsequent sample would also show bacterial growth.

Control of bacterial contamination

During the first part of the study, bacterial growth sometimes occurred in such a way that it was suspected of being contamination. Due to the appearance of this bacterial growth, the aseptic techniques used for collecting the primary material and for production of the media was improved during later stages of the study. These procedures divided the study into three parts (I—III).

A. COLLECTION OF MATERIAL FROM THE DENTINE

Part I (Teeth with working designation nos 37—75): The dissections of the teeth were performed in a box made of transparent plastic without the two end-walls. The stereomicroscope was placed with the objective in contact with the shelter of the box. The inside of the box was disinfected twice at half hour intervals with an aqueous solution of 70 per cent ethanol and 1 per cent quaternary ammonium compound (Colgon, Colgafabriken AB, Stockholm, Sweden) before dissection of the tooth. All the instruments brought into the area were sterilized. The operator wore sterile gloves and arm wristlets. The work was performed through the open end-walls of the box. As soon as the operator had homogenized the dentine in the mortar, it was passed to the assistant who inoculated the substrates by the usual microbiological technique.

Part II (Teeth with working designation nos 76—97): During this part a Laminar Flow Work Station (Microflow LTD, Fleet, Hants, England; Cat. no. 10274) was used to decrease the environmental microbial contamination (Wahlström 1966, Ahrensburg-Christensen and Kristensen 1968, Favero and Berqvist 1968, McDade *et al.* 1969, Cullimore 1970). Both dissection and inoculation of media were performed in the station. The dissection was made as described previously and the inoculation by usual microbial methods considering the modifications which are necessary when working in horizontal laminar air flow (Ahrensburg-Christensen and Kristensen 1968, Heuring 1970). To avoid bacterial contamination from the technician's hands, Savlon antiseptic cream (ICI, LTD, U.K.) was applied to the skin.

Part III (Teeth with working designation nos 99—126): When working in the Laminar Flow Work Station (LFWS), both the operator and the assistant wore sterile gloves and arm wristlets. All equipment was sterilized except material such as plastic petri dishes which were disinfected with 2 per cent (w/v) chlorhexidine in 70 per cent ethanol.

Instead of pipettes, injection syringes with blunt-ended cannulas (length 80 mm, internal diameter 1.2 mm) were used. All the blood agar plates used were prepared in the LFWS.

B. BACTERIAL CONTAMINATION OF THE BLOOD AGAR PLATES APPEARING AFTER THE PRODUCTION OF THE PLATES

In order to study the bacterial contamination of the blood agar, the following two techniques were used during a period of eleven months:

1. Approximately half of all the blood agar plates produced at the laboratory were made in the LFWS. A retort (Fig. 2: 2) was used for the distribution of the substrate into the dishes in the LFWS. The technician wore sterile gloves and arm wristlets.
2. The rest of the blood agar plates were produced as follows: All petri dishes were placed on a working bench in a closed room. From a 1000 ml retort approximately 20 ml of blood agar was poured into each of about forty dishes followed by flaming of the agar surface. The technician did not wear gloves or arm-wristlets. From each batch of forty plates, four or eight plates were randomly selected and incubated at $+37^{\circ}\text{C}$ for four days, two (four) plates in 94 % H_2 and 6 % CO_2 and two (four) aerobically. After incubation the plates were studied under stereomicroscope in both transmitted and reflected light. Observed CFU were isolated. Identification was made by the methods described below.

C. THE EFFECT OF THE INOCULATION PROCEDURES ON THE PREVALENCE OF CONTAMINATING BACTERIA ON BLOOD AGAR PLATES

The following sterility tests were done on four blood agar plates produced in LFWS. Two randomly selected plates were immediately incubated for four days anaerobically and two plates were incubated aerobically without moving the lid from the dish before incubation. The other plates were "inoculated" by streaking the agar surfaces six times with a sterilized platinum loop. The lid of the dish was replaced between each streaking. The inoculation of the plates was performed in ordinary laboratory environment.

D. STERILITY CONTROL OF THE PRODUCTION OF TA-SER MEDIUM

After the addition of serum to the TA-medium (Möller 1966) all the tubes were incubated at $+37^{\circ}\text{C}$ for 2—3 days. From each batch, every 15th tube was selected for further incubation for 28 days. In case of growth identification was made by the method described below.

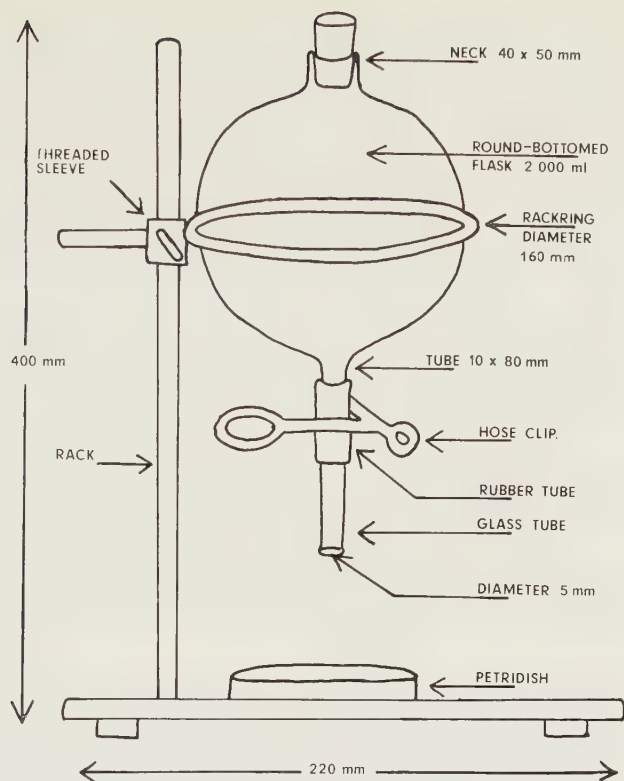


Fig. 2: 2. Retort for production of blood agar plates in the Laminar Flow Work Station.

Preliminary identification of isolates and bacterial contamination

A. TEST METHODS FOR IDENTIFICATION OF BOTH ISOLATES AND BACTERIAL CONTAMINATION

The working isolates were maintained on blood agar and in TA-Ser medium. They were stored in a refrigerator ($+6^{\circ}\text{C}$) and subcultured every second week.

For the tests, the isolates were inoculated on freshly prepared blood agar poured into test tubes—the medium settled in sloped form. However, some of the isolates gave a scant growth on blood agar. These strains were inoculated in TA-Ser medium without agar. Depending on their growth rate during the primary isolation, the isolates were incubated in 94 per cent H_2 and 6 per cent CO_2 (subsequently indicated as $\text{H}_2\text{—CO}_2$) for both the agar and broth medium for 24—72 hours. The cells used as inoculum were washed once in 0.4 per cent NaCl-

solution with 0.02 M K-phosphate buffer (pH 6.8) (Cruickshank 1965).

Unless otherwise stated the broth media were distributed into screw-capped test tubes. Incubation temperature was $+37^{\circ}\text{C}$, and the tests were read on the second, fourth, seventh and fourteenth day.

Colonial morphology and relation to oxygen were studied in the following way. One loopful of the washed inoculate was streaked on blood agar and incubated both in $\text{H}_2\text{—CO}_2$ for 2—6 days, and aerobically for 7 days. Before these tests each isolate was transferred at least twice on blood agar plates incubated in $\text{H}_2\text{—CO}_2$ and maintained in an aerobic atmosphere for more than 48 hours between each transfer. The resulting colonies were examined under a stereomicroscope and described according to Lamanna and Mallette (1963). Those isolates which did not grow in an aerobic atmosphere were further tested with the gas requirement test as described by Slack *et al.* (1963) on Brain-Heart Infusion agar slants and blood agar slants.

Cell morphology was determined as follows. Smears from cultures grown 24—48 hours in TA-Ser medium and blood agar incubated 48—96 hours in $\text{H}_2\text{—CO}_2$ were Gram stained according to the method of Preston and Morell (1962).

Catalase and oxidase tests were run on cultures grown on Brain-Heart Infusion agar (Difco 0037-01) supplemented with 0.1 per cent (w/v) soluble starch (Merck) (subsequently called BHI-agar). The plates were incubated 2—4 days in $\text{H}_2\text{—CO}_2$ followed by further incubation for 2 days aerobically (Roth and Thurn 1962, Cowan and Steel 1965). Positive and negative controls according to Cowan and Steel (1965) were run. The catalase reaction was examined in stereomicroscope.

Production of gas in CTA-medium (Baltimore Biological Laboratories) was studied according to Carlsson (1965). Fermentation of glucose was registered. Positive control strain for the gas test was *Lactobacillus fermentum* NCTC 1750, negative control strain was *Actinomyces eriksonii* ATCC 15423.

The test for capacity to attack sugar by oxidation or fermentation, the O-F-test, was carried out according to Cowan and Steel (1965). The composition of the media followed the recommendation given in the Subcommittee on Taxonomy of Staphylococci and Micrococci (1965). Positive control strains were *Staphylococcus aureus* NCTC 8532 and *Micrococcus spp.* NCTC 7729.

Nitrate reduction was tested in the following medium: Lab-Lemco (Oxoid L30) 10 g; Trypticase (BBL 02-148) 10 g; NaCl 5 g; KNO_3 1 g; distilled water made up to one litre; adjusted to pH 7.2; distributed in screw-capped test tubes in 5 ml amount and autoclaved at $+115^{\circ}\text{C}$ during 20 minutes. The inoculum consisted of 2 drops from a 1 ml

pipette. The nitrate test was made on the seventh day (Cowan and Steel 1965). Positive control strain was *Actinomyces israelii* ATCC 10048, negative control strain was *Streptococcus mutans* NCTC 10449.

One loopful of the washed inocula was streaked on the following four selective media:

1. Mitis-Salivarius agar (Difco B 298); the test was performed according to Edwardsson (1968). Positive control strain was *Strep. mutans* NCTC 10449, negative control strain was *Staphylococcus aureus* NCTC 8532.
2. Staphylococcus Medium No. 110 (Oxoid CM 145). The plates were observed for growth after 24 hours aerobic incubation. Positive control strain was *Staph. aureus* NCTC 8532, negative control strain was *Strep. mutans* NCTC 10449.
3. Blood-tellurite agar according to Cowan and Steel (1965), final tellurite concentration was 0.04 per cent (w/v). The result was read after incubation aerobically for 4 days. Growth and reduction of tellurite were registered. Positive control strain was *Corynebacterium hofmanni* NCTC 231, negative control strain was *Actinomyces israelii* ATCC 10048.
4. Bacto Rogosa SL-agar (Difco 0480-01) according to Rogosa *et al.* (1951). The fresh medium was distributed in 10 ml amounts into test tubes with Kapsenberg caps and inoculated by stabbing deep into the agar. Growth deep in the medium might indicate an anaerobic isolate and was specially registered. Positive control strain was *Lactobacillus fermentum* NCTC 1750, negative control strain was *Actinomyces israelii* ATCC 10048.

Identification was based mainly on the Gram reaction, catalase activity, the ability to grow in air, the reaction in the O-F-test (Cowan and Steel, 1965) and growth on Mitis-Salivarius agar and in Rogosa SL-agar.

ADDENDUM OF TEST METHODS FOR THE IDENTIFICATION OF BACTERIAL CONTAMINATION

Gram positive, catalase positive rods were tested for spore formation. They were grown on starch agar (Cowan and Steel, 1965) in the atmosphere giving the best growth (Slack *et al.* 1968), then placed at room temperature for 5—7 days and stained with the modification of Moeller's method (Cowan and Steel, 1965). However, the ability of the positive control strain to form spores varied and during the later part of the study the isolates were cultured on blood agar. Colonial material was

harvested and placed in a sterile empty dry petri dish at room temperature. After 7 and 14 days the culture was stained for spores (G. Frostell, personal communication, 1971). Cultures of *Bacillus subtilis* ATCC 6633, *Lactobacillus fermentum* NCTC 1750 and *Propionibacterium acnes* NCTC 737 were used as positive and negative controls.

Gram positive, catalase positive, anaerobic rods were also studied for hydrolysis of tributyrin and indol production. Tributyrin hydrolysis was studied on the medium described by Marks (1952) supplemented with 0.2 per cent (w/v) soluble starch and 0.1 per cent (w/v) glucose. The cultures were incubated 7H. The positive control strains included are found in Table 2: 6. *Actinomyces viscosus* ATCC 15987 was included as negative control. Indol production was studied in a semisolid medium with the following composition: Bacto Tryptone 2.0 per cent (w/v), Na_2HPO_4 0.2 per cent (w/v), glucose 0.1 per cent (w/v), Bacto Agar 0.1 per cent (w/v). The medium was sterilized by autoclaving at $+118^\circ\text{C}$ for 15 minutes in 7 ml amounts. The medium was heated to boiling before inoculation. The reaction was read after 3 and 7 days with Kovác's reagent (Cowan and Steel 1965). The control strains were the same as for tributyrin hydrolysis.

Selection of isolates for further characterization

During the primary identification it was observed that the majority of the isolates collected from the blood agar plates incubated 2 days aerobically (2A) and in TA-Ser medium (T) belonged to groups II and III as presented in Table 2: 9. All of these strains could not—for technical reasons—be studied thoroughly by all the tests described in the following chapters. Thus, twelve group II-isolates were tested for gas production from glucose and gluconate, deamination of arginine, growth at $+15^\circ\text{C}$ and $+45^\circ\text{C}$ and hydrolysis of esculin and hippurate as described in Chapter 4. Nine group III-isolates collected from TA-Ser medium (T) were studied with the characteristics used by Mejäre and Edwardsson (1975). The identification of these 21 isolates is presented in Tables 2: 10 and 2: 11. In Table 8: 1 (Chapter 8) the group II-isolates are described as *Lactobacillus spp.*

All the other isolates were identified as described in the following chapters.

RESULTS

Disinfection of the tooth surfaces before sampling

The results of the effect of the iodine varnish as a disinfectant on the tooth surfaces are found in table 2: 1. When the samples were taken from the clinically intact enamel, growth was observed in two cases. On the surface of carious lesions, 40 out of 46 showed growth, while in the decalcified soft lesions growth was observed in 44 cases.

Table 2: 1. *Effectiveness of iodine-varnish (Kelstrup 1966) in disinfecting different surfaces of extracted teeth.*

Sampling surfaces	Number of surfaces with growth
I	2
II	40
III	44

Number of investigated surfaces (n) = 46.

Sampling surfaces

After mechanical removal of the varnish coat and rinsing of the area the following surfaces were tested:

- I Clinically intact surfaces of enamel.
- II Surfaces of carious lesions.
- III Parts of the decalcified lesion (II) were enucleated and the samples taken from the deeper part of the lesion.

Sampling technique, culture media and incubation procedures

In the main study, 87 teeth were investigated. Of these teeth 46 fulfilled the prerequisites (p. 22) for further studies. The growth on each plate and in each TA-Ser medium for every portion of material from all the teeth analysed is presented in Table 2: 2,* pp. 40—41. Some of the figures in the table are indicated in brackets. These figures are considered to represent bacterial contamination and are not further considered here. The contamination problem will be discussed on pp. 34—37.

In table 2: 2 it can also be observed that the depth of the bacterial invasion into the dentine varied. In some cases, growth was found already

* Because of technical reasons at the printing, Table 2: 2 had to be placed at pp. 40—41.

Table 2: 3. *A summary of the results presented in Table 2: 2. Bacterial growth within each portion of dentine.*

	A	B	C	D	E	F
Number of portions with bacterial growth derived from dentine	0	16	32	37	23	5
Number of analysed portions of material	46	46	46	44	25	5
Relative number with growth (percentage)	0	35	70	84	92	100

Explanations see Table 2: 2.

in the B-portions (for example teeth nos. 38, 40, 46 and 48) while in other cases growth did not appear until in the E-portions (teeth nos. 64, 72, 122 and 124).

Table 2: 3 summarizes the bacterial growth within the A, B, C, D, E and F-portions of the 46 teeth further analysed. The first sample (A-portion) from the clinically intact pulpal wall contained no cultivable bacteria. Among the B-portions, bacterial growth was found in 16 cases (35 %) of the teeth analysed. In the subsequent portions C, D, etc., there is an increase in the relative number of cases with bacterial growth.

In 91 per cent of the TA-Ser medium growth appeared, while a smaller number of positive samples were observed for the blood agar plates (7H) (Table 2: 4). This difference between the number of samples with visible growth is statistically significant (Chi-square analysis with Yates' correction, $p < 0.01$). This was also the case when blood agar plates incubated 2A and 7H respectively were compared ($p < 0.001$). Anaerobic incubation gave a numerically higher number of positive samples than aerobic incubation but the difference was not statistically significant ($p > 0.1$).

Table 2: 4 also summarizes the range of variation for the number of CFU observed on the blood agar plates. Judging by the mean figures anaerobic incubation (2H) gave about twice as many CFU as aerobic incubation (2A). The number of CFU further increased with the prolongation of the anaerobic incubation (from 2H to 7H). Irrespective of the weight of the portion in the sample following the one analysed, the number of CFU was on the average four to five times larger. The number of morphologically different CFU observed on the blood agar plates in relation to the incubation procedures are given in table 2: 5. The highest number of CFU was found on the blood agar plates in-

Table 2: 4. Summary of the results in Table 2: 2. Growth intensity on the different media in relation to incubation procedures. Results from the analysed portion (I) and the following portion (II).

	Incubation procedures*							
	2A		2H		7H		T	
	I	II	I	II	I	II	I	II
Numbers of media with visible growth:	16	32	23		35	34	43	44
Per cent of teeth	37	78	50		80	78	98	96
Number of CFU per plate:								
Mean:	55	342	129		591	145	709	
Median:	24	104	23		315	37	468	
Range:	1-202	1-1698	1-630		1-2616	1-1348	1-cg	

* Explanation see Table 2: 2. cg: Confluent growth.

Table 2: 5. *Number of different morphological CFU on the blood agar plates in the analysed portions in relation to incubation procedures.*

Number of different morphological CFU	Incubation procedures*		
	2A	2H	7H
1	7	10	12
2	7	5	6
3	2	5	5
4		2	4
5		1	2
6			2
7			3

* Explanations see Table 2: 2.

cubated 7H where the range is 1—7; corresponding figures for blood agar incubated 2A and 2H are 1—3, and 1—5, respectively.

Further 41 teeth were studied but the results did not fulfil the pre-requisites. These teeth were excluded from the study by the following reasons:

1. Growth was only observed in the last portion 28 teeth
2. Growth did not appear in the last portions, but was found irregularly distributed among the other samples and was suspected for being contamination 7 teeth
3. No growth was observed from any of the dentine portions cultured 6 teeth

Sample size

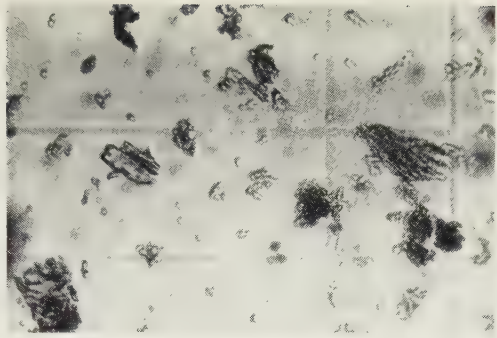
As calculated in the separate series of teeth the mean weight of the dried portions of dentine was 2.72 ± 0.74 mg, the median 2.69 mg and the range 1.53—4.69 mg. The mean size of the dentine chips was $19.5 \mu\text{m}$, the median $9.0 \mu\text{m}$ and the range 1.8—163.8 μm (Fig. 2: 3).

Control of bacterial contamination

A. COLLECTION OF MATERIAL FROM THE DENTINE

During part I (teeth with nos. 37—72 in Table 2: 2) heavy growth sometimes appeared on one of the plates in one portion but no growth was

Fig. 2:3. The configuration of dentine chips after homogenization in glass mortar and staining with Loeffler methylene blue solution. Magnification $\times 400$. White bar in lower right corner represents 10 μ m.



observed on the other plates or in the TA-Ser medium in the same portion. This is illustrated by tooth no. 47 portion B. In other cases single CFU sometimes appeared on one of the plates in one portion but no growth was observed on the other plates in the same portion and in the subsequent portion. An example is tooth no. 43, portion A. Isolates from these CFU were identified mainly as *Staphylococcus*-like microorganisms and *Propionibacterium*-like microorganisms. Characteristics of isolates resembling those of *Propionibacterium* are found in Table 2: 6.

Improvements in sampling technique resulted in a decrease of these types of CFU from 5.8 per cent of the blood agar plates during part I to 4.8 per cent during part II of the study. During part III, no CFU of these bacteria were observed; the results are summarized in Table 2: 7.

B. BACTERIAL CONTAMINATION OF THE BLOOD AGAR PLATES APPEARING AFTER THE PRODUCTION OF THE PLATES

In the Laminar Flow Work Station 1383 blood agar plates were produced, of which 27 per cent were incubated as controls. Five plates (1.3 per cent) were observed to be contaminated with *Staphylococcus epidermidis* in 2 cases, *Bacillus spp.* in one case and with *Propionibacterium/Corynebacterium* in 2 cases.

Of the blood agar plates (1277) produced by the usual technique in an ordinary laboratory environment, 14 per cent were incubated as controls and 15 plates (8.7 per cent) were found to be contaminated with *Staphylococcus spp./Micrococcus spp.* in 8 cases, *Corynebacterium spp./Propionibacterium spp.* in 4 cases, *Bacillus spp.* in one case and psychrophilic Gram negative rods in 2 cases.

Table 2: 6. *Characteristics of 21 Propionibacterium-like isolates suspected of being contaminants.*

Gram positive pleomorphic rods								Characteristics of reference strains			
Characteristics ²	Number of isolates							Propionibacterium acnes			
	12	3	2	1	1	1	1	ATCC 6921	ATCC 6922	NCTC 737	Serotype II (Voss 1970)
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-
CTA-medium:											
motility	-	-	-	-	-	-	-	-	-	-	-
fermentation of glucose	+	+	+	+	+	+	+	+	+	+	+
O-F-test:											
fermentation	+	+	+	+	+	+	+	+	+	+	+
oxidation	-	-	-	-	-	-	-	-	-	-	-
Mitis Salivarius agar	-	-	-	-	-	-	-	-	-	-	-
Rogosa: Selective Lactobacilli agar	+	+	- ¹	- ¹	+	- ¹	- ¹	- ¹	- ¹	+	+
Blood-tellurite agar 0.04 %	-	-	-	-	-	-	-	-	-	-	-
Nitrate-reduction	+	+	+	+	-	-	+	+	+	+	+
Staphylococcus Medium 110	-	-	±	-	-	-	-	-	-	-	-
Tributyrin, hydrolysis of:	+	+	+	+	+	+	+	+	+	+	+
Indol production	+	+	+	+	-	-	+	+	+	+	-
Blood agar aerobically	-	-	±	±	-	-	-	-	-	-	-

¹ All the isolates were positive after 7—14 days.

² None of the isolates formed spores.

C. THE EFFECT OF INOCULATION PROCEDURES ON THE PREVALENCE OF CONTAMINATING BACTERIA ON BLOOD AGAR PLATES

The effect of "inoculation" streaks on the prevalence of contaminating bacteria after incubation anaerobically and aerobically was studied on blood agar plates produced in LFWS and selected for the sterility test as described above. Blood agar plates (4460) were produced and 308 (7 per cent) selected for the control. The results are shown in Table 2: 8. When the plates were incubated immediately after production without moving the lid from the dish (without any inoculation procedures), it

Table 2: 7. *Review of the techniques used for the control of the environmental microbial contamination and the observed prevalence of suspected contaminations on agar medium during the three parts of the study.*

Part	Collecting of material		Blood agar produced in environment	Number of teeth investigated		Prevalence of suspected contamination	
	Working environment	Operator (dissection of tooth)	Technician (inoculation of substrates)	Total	With growth in any of the portions	Number of blood agar plates used:	With suspected contamination
I	Plastic box	Aseptic	Usual technique	37	84 %	516	5.8 %
II	LFWS ¹	Aseptic	Usual technique + antiseptic handcream in LFWS	23	87 %	330	4.8 %
III	LFWS	Aseptic	Aseptic in LFWS	27	85 %	348	0 %

¹ LFWS: Laminar Flow Work Station.

Table 2: 8. *Prevalence of contamination on blood agar plates incubated as sterility test.*

Observed contamination on the plates identified as	Number of contaminated plates incubated			
	Without any inoculation- procedures		After inoculation with sterilized platinum loop	
	Anaerobic	Aerobic	Anaerobic	Aerobic
<i>Staphylococcus epidermidis</i>		1	7	3
<i>Micrococcus spp.</i>			1	1
<i>Propionibacterium spp.</i>	1		3	
Gram negative rod (psychrophilic)				1
Total number of blood agar plates incubated as control:				
	77	77	77	77
Contaminated	1.3 %	1.3 %	14 %	6.5 %

was observed that 1.3 per cent of the plates were contaminated. On the other hand, when “inoculation” streaks were made, 14 per cent of the anaerobically and 6.5 per cent of the aerobically incubated plates were contaminated. Isolates resembling *Staphylococcus epidermidis* were most frequently observed.

D. STERILITY CONTROL OF THE PRODUCTION OF TA-SER MEDIUM

During part I—III of the study about 10,000 TA-Ser medium tubes were prepared. Sixhundred eighteen tubes were selected as controls and were incubated for 30 days. In 12 tubes (2 per cent) growth appeared. Isolates from 10 tubes were found to resemble genus *Propionibacterium* and in two cases *Staphylococcus*-like isolates were observed. The characteristics of these *Propionibacterium*-like rods were close to those given for other *Propionibacterium*-like isolates presented in Table 2: 6.

When preparing the TA-Ser medium, the TA-medium was autoclaved according to the recommendation of the manufacturer (Difco Laboratories, Detroit; 15 minutes at 15 pounds pressure, + 121°C). However, it has been observed that this procedure may not give sterility in thioglycollate media (Tiru, 1971, personal communication). Therefore the TA-medium was autoclaved for 40 min. at + 120°C. About 10,000 tubes of TA-Ser medium have been prepared in this way using prolonged sterilization time. Out of these, 693 tubes (7 per cent) were tested for

sterility as described above. In one tube (0.1 per cent) growth was observed. The isolate from this tube was found to be a *Propionibacterium*-like microorganism.

Preliminary identification of isolates from dentine

A total of 220 isolates were collected. Three of these isolates primarily found on blood agar failed to grow after transfer to TA-Ser medium. These isolates appeared as Gram positive pleomorphic rods in smears taken from the blood agar. Another two coccus-like isolates were lost before final examination. All the other isolates could be recultured and were tentatively placed in one of the groups described below (Tables 2: 9—11).

Group I

Seventy isolates were found to be Gram positive pleomorphic rods.

Four of the isolates were able to grow on the tellurite blood agar forming black colonies. Fifty-six of the isolates were catalase negative. Forty-seven isolates fermented glucose in the CTA- or O-F-media. Eighteen reduced nitrate. Twelve isolates were able to grow on the blood agar plates after 7 days aerobic incubation. When the oxygen requirement tests (Slack *et al.* 1968) were performed another five isolates were observed to grow aerobically.

They were separated from the isolates in group II by their pleomorphic appearance in Gram stains and inability to grow out in Rogosa SL-agar within 4 days except for four catalase positive isolates.

Group II

This group consisted of ninety-six Gram positive rod-like organisms, which mainly appeared with straight, squared, short to long rods; curved cells were also observed while filaments occurred seldomly. All in this group were able to grow out in Rogosa SL-medium within 48 hours. Eighty-two of the isolates fermented glucose in the O-F-test. Only seven produced gas in CTA-medium. Of the isolates, all grew on blood agar incubated aerobically with CO₂ (Slack *et al.* 1968). However, as a rule, growth was more luxurious in an anaerobic atmosphere. None of the isolates were motile or produced catalase, nor did any of them reduce nitrate.

Table 2: 9. Preliminary assignment of the isolates into groups based on some easily determined characteristics.

Characteristics	Group I Gram positive pleomorphic rods Number of isolates		Group II Gram positive rods Number of isolates		Group III Gram positive cocci Number of isolates		Group IV Gram negative organisms Number of isolates		Group V Gram variable cocci/coccoid rods Number of isolates	
	70 33 %	96 45 %	28 13 %	6 2 %	3 1 %	2 1 %	6 3 %	5 2 %	anaerobic	facultative anaerobic
Catalase	15	0	0	2	0	0	0	5	0	5
Oxidase	0	0	0	0	0	0	0	0	0	0
CTA-medium:										
Motility	0	0	0	0	0	0	0	1	0	1
Fermentation of glucose	47	82	28	0 ²	1 ³	0	2	4	0	4
Gas-production	0	7	0	0	0	0	0	0	0	0
O ₂ -test:										
Fermentation	48	85	28	0 ²	0	0	0	4	0	4
Oxidation	0	0	0	0	0	0	0	1	0	1
Mitis-Salivarius agar	5	69	28	0	0	0	0	0	0	0
Rogosa SL agar	4 ¹	96	9	0	0	0	0 ⁴	0	0	0
Blood-tellurite										
agar 0.04 %	4	0	11	0	0	0	0	2	0	2
Nitrate-reduction	18	0	0	0	0	0	0	3	0	3
Staphylococcus Medium										
No. 110	2	0	1	0	0	0	0	4	0	4
Aerobic blood agar	12	88	28	0	0	0	0	5	0	5
Oxygen dependence:										
growth aerobic	17	94	NT ³	0	0	0	0	5	0	5
growth aerobic + CO ₂	17	96	NT	0	0	0	0	5	0	5

¹ Another 13 isolates grew after 7-14 days incubation mainly deep in the medium.

² All isolates reduced the colour indicator after 7-14 days incubation.

³ One isolate reduced the colour indicator after 10 days incubation.

⁴ Four isolates showed meagre growth deep in the agar after 14 days incubation.

⁵ NT not tested.

Table 2: 10. *Tentative identification of twelve group II-isolates resembling Lactobacillus spp., collected from blood agar incubated aerobically (2A) and TA-Ser medium (T).*

Characteristics studied:	Isolates assigned to <i>Lactobacillus</i> subgenera		
	Thermo- bacterium 1 ¹	Strepto- bacterium 10	Beta- bacterium 1
Gas from			
glucose	0 ²	0	1
gluconate	0	10	1
Deamination of			
arginine	0	0	0
Hydrolysis of			
esculin	1	10	1
hippurate	0	1	1
Growth at			
+ 15°C	0	9	0
+ 45°C	1	2	1

¹ Number of isolates assigned to respective subgenera.

² Number of isolates being positive in respective test.

Table 2: 11. *“Key”-characteristics for identification of nine group III-isolates resembling Strep. mutans, Strep. milleri and Strep. mitior isolated from TA-Ser medium (T).*

Characteristics	Strep. mutans 2 ¹	Strep. milleri 3	Strep. mitior 4
Extracellular polysaccharide			
from sucrose	2 ²	0	0
Production of H ₂ O ₂	0	0	4
Arginine deamination	0	3	2
Growth on MC-agar	2	3	0
Acid from			
mannitol	2	0	0
sorbitol	2	0	0
Sensitive to:			
bacitracin (0.2 IU/disc)	0	0	4
sulfisodimidine	0	0	4

¹ Number of isolates resembling respective species.

² Number of isolates giving a positive reaction.

By using the experience of the study by Mejåre and Edwardsson (1975), the above given tests could be described as “key”-characteristics for the identification of these nine *Streptococcus*-like isolates. The identification was therefore based mainly on the results in these tests and on the colonial morphology on Mitis-Salivarius agar.

Table 2: 2. Growth intensity from each portion of dentine. Results from 46 teeth where further analyses were performed in order to identify the bacterial isolates.

A			B			C			D			E			F					
2A	2H	7H	T	2A	2H	7H	T	2A	2H	7H	T	2A	2H	7H	T	2A	2H	7H	T	
37	-	-	(1/1) ^s	-	-	-	(1/1) ^p	-	-	-	+	1	1	632	+	42	101	369	+	
38	-	-	-	-	-	1/1	-	1	1	1	-	73	85	237	+	25	321	573	+	
40	-	-	-	-	-	7/4	+	77	254	1237	+	cg	cg	cg	+					
42	-	-	-	-	-	-	-	-	-	-	+	638	1436	2148	+					
43	-	-	(1/1) ^p	-	-	-	-	-	-	-	+	-	-	-	+	127	153	2136	+	
44	-	-	-	-	-	-	-	-	-	-	-	23/1	34/1	34/1	+	30	34	33	+	
46	-	-	(1/1) ^p	-	-	1/1	+	-	-	1	+	954	1556	2460	+	1580	2000	cg	+	
47	-	-	-	-	(348) ^s	-	-	-	14/1	89/4	-	-	4	25	+					
48	-	-	-	-	-	-	+	400	2616	cg	+	+	+	+	+					
49	-	-	-	-	5/1	1/1	+	11	12	23	+	824	926	908	+					
50	-	-	-	-	-	-	+	-	113	204	+	5	723	cg	+					
51	-	-	-	-	-	-	-	-	-	-	+	-	-	57	+					
55	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	150	347	312	+	
58	-	-	-	-	-	7/2	+	88	76	89	+	5	11	11	+					
62	-	-	-	-	-	-	-	-	(1/1) ^s	(1/1) ^p	-	3/2	6/2	8/3	+	2	5	6	+	
63	-	-	(1/1) ^p	-	-	-	-	-	-	-	-	-	-	2/2	+	3	14	97	+	
64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	204/3	+	
68	-	-	-	-	-	-	(1/1) ^p	-	-	2/1	446/6	+	160	1354	2118	+	526	524	1946	+
72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8/1	+	
77	-	-	(1/1) ^p	-	-	-	-	-	-	-	-	-	-	-	+	-	12	143	+	
78	-	-	-	-	-	-	-	-	23/2	40/1	+	513	912	1316	+	+	+	+	+	
79	-	-	-	-	-	2/1	-	-	-	9/1	+	-	125	320	+					
80	-	-	-	-	-	-	-	-	-	4/1	+	-	-	574	+	-	436	632	+	
81	-	-	-	-	81	-	-	-	1/1	-	+	1058	1346	2630	+					
82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	528	614	1440	+	
85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	120	1824	cg	+	

102	-	-	-	30/1	29/1	57/2	+	942	1008	1728	+	+	+	+	+	+	+	+	+
103	-	-	-	-	-	-	-	109/2	198/4	105/4	+	526	548	1756	+	+	+	+	+
104	-	-	-	-	-	-	-	-	3/3	253/7	+	1	19	300	+	+	+	+	+
106	-	-	-	202/2	514/3	734/3	+	1698	1882	2324	+	+	+	+	+	+	+	+	+
108	-	-	-	-	4/1	8/2	+	73	76	116	+	+	+	+	+	+	+	+	+
109	-	-	-	-	-	-	-	-	-	-	-	24/1	31/2	119/3	+	486	942	858	+
110	-	-	-	1/1	2/1	1/1	+	-	-	2	-	-	-	-	-	-	-	-	-
111	-	-	-	-	-	-	-	13/1	160/3	117/6	+	1	21	56	+	1	6	1	+
113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	4	+
115	-	-	-	7/3	9/4	17/5	+	1	-	3	+	-	-	-	-	-	-	-	-
116	-	-	-	-	-	-	-	-	-	-	-	147/3	226/6	183/7	+	952	1434	1892	+
119	-	-	-	-	-	-	-	-	-	-	-	-	-	54/3	+	-	-	528	+
122	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	202/1	+
124	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	408/1	209/1	+
125	-	-	-	-	-	-	-	72/2	105/3	129/7	+	84	201	382	+	+	+	+	+
126	-	-	-	-	-	-	-	-	-	-	+	342	520	568	+	+	+	+	+

A—F

Designation for the portions of material, portion A always contained material from the dentine wall of the pulp.
(See also Fig. 2: 1.)

2A, 2H, 7H

Material inoculated on blood agar and incubated 2 days aerobically, 2 days and 7 days in 94 % H₂ + 6 % CO₂, respectively.

T:

Material inoculated in TA-Ser medium and incubated for 14 days.

Working designation of the teeth.

Growth in TA-Ser medium.

No growth.

Confluent growth.

Figure 37—126 (left

vertical column):

+

-

cg:

The figures represent the number of CFU on each plate. In the portions analysed the two figures represent: number of CFU/number of different morphological CFU. Figures within brackets are considered to represent contamination; (n)p respectively (n)s= isolates from colonial material resembling *Propionibacterium* and *Micrococcaceae* respectively.

Group III

This group consisted of twenty-eight Gram positive cocci which appeared in chains, in smears from TA-Ser medium. All the isolates were non-motile and catalase negative. All of them fermented glucose and grew well on Mitis-Salivarius agar and blood agar aerobically.

Group IV

Nine isolates were found to be Gram negative anaerobic cocci and rods. Six of these isolates were observed to be cocci with the size varying from 0.6—0.9 μm ; the cells often appearing in masses. None of them utilized glucose but reduced the colour indicator both in the O-F-medium and the CTA-medium. Two of the isolates were able to decompose hydrogen peroxide.

Three isolates were observed to be Gram negative non-motile rods, two of them appearing with crescent formed shape. They only grew to any notable extent in TA-Ser medium and on blood agar anaerobically and was not stimulated by CO_2 in the tests according to Slack *et al.* (1968).

Group V

This group consisted of eleven isolates. One of the isolates showed a distinct coccus-like cell morphology, grew aerobically, was catalase positive, oxidized glucose and reduced nitrate.

Six isolates were found to be Gram variable pleomorphic cocci or coccoid rods which did not grow on blood agar aerobically or were stimulated by CO_2 in the anaerobic environment. Two of these isolates fermented glucose in CTA-medium; the acid reaction became visible after 10 days. In SL-agar, four isolates showed growth deep in the medium after 7—14 days.

The results of the identification of certain group II- and group III-isolates, which are not further characterized in the following chapters, are given in Tables 2: 10 and 2: 11.

DISCUSSION

Clinically intact dentine beneath carious lesions is considered not to harbour any microorganisms even at rather small distances from the lesion (Miller 1891, Stephan *et al.* 1943, MacGregor *et al.* 1956, Fusayama *et al.* 1966). The material in this study was collected by approaching the lesion from the pulpal wall of the dentine with the intent of isolating the cultivable bacterial flora of the innermost part of

the lesion and of avoiding the abundant biota of the fully developed lesion. The first portion taken from the pulpal wall (the A-portion) should not contain any cultivable bacteria. If this were the case it might be questioned whether the observed bacterial growth represents the front line of cultivable bacterial invasion or if this front line already has passed into pulpal tissue and the bacterial growth represents the flora of the lesion in a more advanced state of decay. Consequently, all the teeth were discarded if the dentine wall beneath the carious lesion was discoloured or soft since such a condition may indicate the presence of bacteria in the region (Onisi and Nuckolls 1954, 1958, MacGregor *et al.* 1956, Fusayama *et al.* 1966).

Following portion A, the first portion (B, C or D-portion etc.) with visible bacterial growth was selected and CFU isolated for further studies. This selection was made on the assumption that this portion represented the cultivable bacterial flora of the innermost part of the decayed dentine. If this assumption were correct, the subsequent portion should represent parts of the lesion in a more advanced state of decay, where the number of cultivable bacteria would be larger than in the deeper zones (Burnett and Scherp 1951 a). Therefore, bacterial growth should be present in the portion following the sample analysed.

The most reliable results are to be expected when the number of cultivable bacteria (CFU) was higher in the subsequent portion than in the portion analysed. Usually this was the case (Table 2: 4). However, the number of organisms in the subsequent portion could be influenced by different circumstances. The amount of dentine removed in the different portions varied with a range of about 1 to 4 mg, and penetration of the surface iodine varnish may have taken place in some instances. Such circumstances could influence the number of cultivable bacteria in this portion, giving rise to a low number of CFU. Therefore, if growth occurred in one portion, it was considered as being derivated from carious dentine even in those cases where few CFU were observed on the media in the subsequent portion.

When isolating the bacterial flora of the deeper part of the dentine carious lesions, it is essential to work with methods that minimize bacterial contamination of the samples from the tooth surface. These problems have been thoroughly discussed by Onisi and Nuckolls (1954, 1958). For decontamination of the tooth surface, iodine varnish according to Kelstrup (1966) was chosen because iodine compounds are effective for disinfecting surfaces of hard dentine (Möller 1966). Kelstrup (1966) also reported that this varnish was only slightly miscible with water which prevents it from diffusing into water-filled spaces. He also found that iodine was not liberated after drying of the varnish. The

result of the separate pilot study also indicated that the iodine varnish had a decontaminating effect on the surface of the intact enamel, without involving the bacterial flora within the lesion to any appreciable degree. Furthermore, during the main study, only the iodine varnish over the pulpal wall of dentine was removed. During the whole collecting procedures all the other parts of the tooth were covered with varnish, which thus further decreased the possibilities of microbial contamination interfering with the bacterial flora in the samples.

The bacterial growth which was suspected as being contamination during the main study (Tables 2:2 and 2:7) and those organisms appearing during the preparation of the blood agar plates and after inoculation procedures (Table 2:8) were mainly identified as *Micrococcaceae* and *Corynebacteriaceae*. This is in agreement with previous observations. Bacteria belonging to these families are normal inhabitants of human skin (Kligman 1965) and the human skin biota is considered as an important airborne contaminating flora, both in the surgical theatre and in the laboratory (Bourdillon *et al.* 1948, Bernard *et al.* 1965, McDade *et al.* 1966 and Cato *et al.* 1970). The presence of *Micrococcaceae*-like and *Corynebacteriaceae*-like organisms on "occasional" plates were therefore considered as bacterial contamination and not as derivated from the dentine. To emphasize omission of such CFU from the rest of the material, they were put in brackets in Table 2:2.

Thus, by approaching the lesion from the pulpal wall of the dentine it became possible to detect and finally to control the bacterial contamination. However, in the portions analysed in part I and II of the study, *Staphylococcus*- and *Propionibacterium*-like microorganisms sometimes appeared. Based on the observation that staphylococci were not observed in part III of the study, when the improvements of the technique were completed, such isolates may be considered as contaminants. On the other hand, *Propionibacterium*-like organisms were found also in part III in two of the portions analysed. Strains resembling *Propionibacterium* have been observed in gingival debris and plaque (Rasmussen *et al.* 1966). For these reasons it is considered that organisms resembling *Propionibacterium* also could be present in the dentine carious lesion. Their existence in this acid environment (Dirksen *et al.* 1963) is supported by the observation that most of these isolates were aciduric (Table 2:6).

After increasing the sterilization time for the TA-medium from 15 to 40 minutes, the presence of bacterial contamination in the TA-Ser medium decreased. Long sterilization times might decrease the growth-promoting capacity of the media (Cowan and Steel 1965, Möller 1966). However, TA-medium autoclaved for 40 minutes and supplemented

with 10 per cent horse serum has been used in the laboratory without any observed decrease or failure of growth of fastidious organisms such as *Eubacterium*, *Selenomonas*, *Peptostreptococcus* or *Veillonella*.

The size of the dentine chips after homogenization in the mortar varied from 1.8 to 163.8 micrometers. Considering this variation it is possible that inside certain chips various amounts of bacteria may be located so that they would not be able to grow out on the media. Therefore, no calculation was performed to establish the amount of microorganisms lost during the inoculation procedures and to establish the number of bacteria per weight or volume. Furthermore, weighting procedures would have increased the exposing of the samples for oxygen which might have decreased the recovery of anaerobic microorganisms (Loesche 1969). Only the relative proportions of different types of CFU on each blood agar plate were calculated as this count was considered to be more independent of the inoculum size and the amount of moisture and inert material present.

During the pilot study for the present work, 6 teeth were completely analysed by the described sampling technique but with less consideration to suitable anaerobic technique. Bacteria which were considered as anaerobic constituted 19 per cent of the isolates. In the main study the corresponding figure was about three times higher and the mean number of CFU on the blood agar plates (7H) had increased about four times. Thus, the procedures to reduce contact of the specimens with the oxygen were effective in increasing detection of anaerobic bacteria. However, the possibility should not be excluded that the application of a strictly anaerobic technique (Hungate 1950, Moore 1966, Cato *et al.* 1970, Aranki *et al.* 1969), would further increase the number of anaerobic bacteria observed. This has been the experience of other studies on the oral bacterial flora (Aranki *et al.* 1969, McMinn and Crawford 1970, Gordon *et al.* 1971).

Using a stereomicroscope, CFU with different morphology were isolated from blood agar plates. Different genera and species residing in the oral cavity may have colonies with approximately the same morphology (Onisi and Nuckolls 1954, Socransky 1970). Therefore, in the isolation several CFU of each type were taken for Gram staining. In almost every case agreement was observed between colony forms and cell morphology. Thus, CFU with the same morphology also contained cells with the same shape. For more than 50 per cent of the analysed portions, this checking meant that almost all CFU present were studied. On the other hand, when a large number of CFU was present on the plates, certain strains may be overlooked, since all the CFU could not be checked. However, when the number of colonies on a solid medium is

high, dissociation forms are more frequent (Dean and Hinshelwood 1966). In such cases in the present study a larger number of CFU was studied.

Omitting suspected contaminated cultures, all the isolates presented in Table 2: 9 were collected from the first portion giving a positive culture. These isolates were considered as being derived from the advancing front of the lesion. The few characteristics studied for each isolate and compiled in Table 2: 9 were mainly used for checking on the purity of lyophilized cultures of the isolates. The grouping described was only considered as a temporary clustering until further information about the isolates was obtained. This is the subject of the following chapters.

SUMMARY

The bacterial flora of deep dentine carious lesions has been studied. Forty-six extracted teeth, with lesions extending into the dentine, were investigated within four hours after extraction of the teeth. The surfaces of the teeth were disinfected with an iodine-ether-colophonium solution. Using an aseptic technique, successive portions of dentine were removed from the pulpal wall towards the lesion. When soft necrotic lesions were reached no further material was sampled. Each portion of dentine was homogenized and inoculated on three blood agar plates incubated 2—3 days and 7 days in 94 per cent H₂ and 6 per cent CO₂ and 2—3 days aerobically. Material was also inoculated in one test-tube containing thioglycollate medium supplemented with horse serum; this tube was incubated up to 14 days.

It was assumed that clinically intact dentine beneath the lesion does not contain cultivable bacteria. The first portion of material to give a positive culture should therefore contain bacteria from the advancing front of the lesion. In this culture the different morphological colony forming units (CFU) were counted and 220 representative CFU isolated, preliminarily identified and then freeze-dried. Based on the preliminary identification, the isolates were brought into five groups (Table 2: 9). Separately twelve group II-isolates and 9 group III-isolates were further studied and identified, Tables 2: 10—11.

By approaching the lesion from the pulp, it was possible to avoid contamination of the samples with bacteria from the soft necrotic material in the superficial layers of carious dentine. This approach also made it possible to detect and finally minimize the airborne bacterial contamination mainly identified as *Micrococcaceae* and *Corynebacteriaceae*.

3. Identification of Gram positive pleomorphic rods

In collaboration with G. H. Bowden, Dental Bacteriology Laboratory, The London Hospital Medical College, Dental School, London E 1, England

At the preliminary identification (Chapter 2), seventy isolates were assigned to group I as Gram positive pleomorphic rods. They varied widely in growth: some of them grew only scantily in the TA-Ser medium and on blood agar anaerobically while others were biochemically more active. But on closer study of these isolates, five of them were found to be Gram positive anaerobic cocci and were transferred to group V and another one found to have characteristics of Gram negative rods and were transferred to group IV. These isolates will be described in greater detail in Chapters 6 and 7.

This chapter, which is a summary of an article by Edwardsson and Bowden (1975), concerns the remaining 64 isolates, all of which were studied with gas chromatographic analysis, cell wall analysis, gel diffusion analysis and biochemical tests.

MATERIAL AND METHODS

Acid end products

The acid end products were analysed with the methods of Bowden *et al.* (1975) and Edwardsson and Bowden (1975).

Cell wall analysis

Cell walls were examined for: lysine, ornithine, diaminopimelic acid (DL. DAP and LL. DAP), glucose, mannose, rhamnose and 6-deoxytalose with the methods described by Hoare and Work (1957), Atfield and Morris (1961), Kaufmann *et al.* (1967), Bowden (1969), Bowden *et al.* (1975) and Edwardsson and Bowden (1975).

Gel diffusion analysis of *Actinomyces*-like and *Rothia*-like isolates was performed with the methods described by Shattock (1949), Bowden and Hardie (1972) and Edwardsson and Bowden (1975).

Biochemical tests

Edwardsson and Bowden (1975) have given details of the methods used for examining the cell morphology seen in Gram stained smears and wet preparations for darkfield and phase contrast microscope, motility, catalase reaction, spore formation, acid production from carbohydrates, hydrolysis of starch, gelatine, tributyrin and esculin, production of hydrogen sulphide, lecithinase, gas and acetylmethylcarbinol (VP-test), reduction of nitrate and nitrite and the presence of a cytochrome system.

Organisms in Group 6 grew only poorly in the standard medium and the biochemical tests were repeated with the media and methods described by Holdeman and Moore (1972).

The results of the biochemical tests are not included *in extenso*. Those characteristics considered of value by other workers for the identification of these organisms (Holdeman and Moore 1972, Cummins and Johnson 1974, Scardovi and Crociani 1974) and which were observed in the present study are reported here. Full details of the results of the biochemical tests are available on request from the authors.

Reference strains

Some of the reference strains are included in Table 3: 2—7. A more detailed description of these strains has been given by Edwardsson and Bowden (1975).

RESULTS AND DISCUSSION

All the 64 isolates were Gram positive, non-motile, pleomorphic rods. None of them produced spores or lecithinase. Further division into genera was based mainly on the results of the gas chromatographic analysis. The isolates were divided into six groups which in general corresponded to six genera (Table 3: 1).

Group 1

Fourteen isolates were assigned to this group (Table 3: 2). Branching and pleomorphic cells detected mainly in wet preparations of all these isolates. The ratio of volatile to non-volatile acid was 1:13 and the cell wall analysis revealed both ornithine and lysine. The pleomorphism and branching cells (Davis *et al.* 1955, Davis 1955, Slack and Gerencser 1970), their inability to grow in Rogosa SL-medium (Rogosa *et al.* 1951) and the presence of both lysine and ornithine in the cell walls (Williams

Table 3: 1. *Grouping of the isolates on basis of acid end products of glucose.*

Group	Ratio of acid production in Meq ¹						Identification
	acetic	propionic	butyric	valeric	caproic	non-volatile	
						No. of isolates	
1	1.0					13.0	14 Actinomyces
2	1.0					1.6	2 Rothia
3	1.0					1.0	14 Bifidobacterium
4	1.0	1.6				0.3	11 Arachnia
5	1.0	2.2				0.6	10 Propionibacterium
6	1.0		0.8	tr ²	1.0		11 (12)* Eubacterium

¹ Calculated as meq/100 ml. of culture and expressed as the ratio of volatile acids and non-volatile acids to acetic acid. Average figure for the whole group.

² tr = trace.

* No data available for one Group VI-isolate.

1971, Schleifer and Kandler 1972) distinguished these isolates from those identified as lactobacilli (Chapter 4). The characteristics of the isolates in Group 1 were consistent with their being *Actinomyces*-like organisms (Holdeman and Moore 1972, Schleifer and Kandler 1972, Bowden and Hardie 1973 a). The composition of carbohydrate components in the cell wall supported the suggestion that some were *Actinomyces*, in particular the finding of 6-deoxytalose together with rhamnose in seven of the strains. Apart from one report of this 6-deoxy-sugar in *Strep. cremoris* (Colman and Williams 1965), it has been detected only in *Actinomyces* (MacLennan 1961, Hammond *et al.* 1973). The majority of the isolates reduced nitrate, hydrolysed esculin and produced acid both from soluble and insoluble starch while none of them produced gas and this agrees with the description of Moore and Holdeman (1970) for *Actinomyces*-like strains.

Four isolates reacted in the gel diffusion test with sera from *A. israelii*, and galactose was detected in the cell wall preparations. These were identified as *A. israelii* (Bowden and Hardie 1973 a). One isolate resembled *A. israelii* in that it produced a weak precipitin line with *A. israelii* serotype 1 NCTC 4860. But this isolate showed a closer relationship to *A. naeslundii* by the presence of rhamnose and 6-deoxy-talose as cell wall components and by the ability to utilize various sugars and was therefore identified as a strain *A. naeslundii* (Howell *et al.* 1959, Slack *et al.* 1969, Bowden and Hardie 1973 a).

Although the acid extracts of two isolates did not react with *A. naeslundii* ATCC 12104 antiserum, these isolates resembled that of *A. naeslundii* in respect of the pattern of cell wall components and their ability

Table 3: 2. Comparison between cell wall components and biochemical characteristics of *Actinomyces*-like isolates and reference strains.

Characteristics of reference strains and isolates resembling respective species										
	<i>A. israelii</i>		<i>A. naeslundii</i>		<i>A. odontolyticus</i>		<i>A. viscosus</i>		<i>A. sp.</i>	
	ATCC 10048	isolates n = 4 ¹	ATCC 12104	isolates n = 3	NCTC 9935	WVU 482	ATCC 15987	WVU 371	isolates n = 3	isolates n = 2
Cell wall components ⁴										
galactose	+ ²	4 ³	-	1	+	+	+	+	3	0
glucose	-	4	+	2	+	+	tr	tr	3	2
mannose	-	1	+	2	tr	tr	-	-	3	2
rhamnose	-	0	+	3	+	+	+	+	3	2
6-deoxytalose	-	0	+	3	+	+	+	+	3	2
Catalase	-	0	-	0	-	-	+	+	3	0
Hydrolysis of										
soluble starch ⁵	-	1	-	2	+	+	-	-	3	2
esculin	+	3	+	1	-	+	+	+	2	1
Reduction of										
nitrate	+	3	+	1	+	+	+	+	3	2
nitrite	-	0	-	0	-	-	-	-	2	0
Acid from										
arabinose	-	1	-	0	+	+	+	-	0	0
cellobiose	+	2	V	0	-	-	-	-	0	0
lactose	+	4 ^V	-	2	+	-	+	+	2	1
maltose	+	4	+	3	+	+	+	+	3	1
mannitol	-	2 ^V	-	0	-	-	-	-	0	0
mannose	-	2	+	2	-	-	+	-	1	0
raffinose	+	3	+	3	-	-	+	+	3	1
sucrose	+	3	+	2	-	+	+	+	3	1
xylose	+	3	V	0	+	-	+	+	2 ^V	0

Symbols

¹ Number of isolates resembling respective species.² + = positive and - = negative reaction for respective reference strains.³ The number of isolates giving a positive reaction.⁴ V designates a variable result; the result given is recorded as that which occurred twice in three tests.

tr trace.

⁵ Ornithine and lysine were detected from all the isolates but not DL-DAP or LL-DAP.⁶ All isolates showed same pattern of hydrolysis of insoluble starch. All isolates produced acid from soluble and insoluble starch but none produced indol or gas, tributyrin and gelatine were not split.

to utilize various sugars. They were therefore identified as *A. naeslundii* (Howell *et al.* 1959, Slack *et al.* 1969, Moore and Holdeman 1970, Bowden and Hardie 1973 a).

Two isolates produced brown-red colonies when grown aerobically on blood agar. One of them reacted also with *A. odontolyticus* serotype 1 antiserum in the gel diffusion analysis, and one of its cell wall components was 6-deoxytalose. This isolate was therefore regarded as *A. odontolyticus* (Batty 1958, Bowden and Hardie 1973 a). The other isolate, which gave growth of brown-red colonies did not react in the gel diffusion analysis, and the cell wall components were not consistent with those described for *A. odontolyticus*. This isolate was provisionally diagnosed as *A. odontolyticus* solely on the demonstration of the colour of the colonies on blood agar (Batty 1958). Three isolates were catalase positive and reacted with *A. viscosus* serotype 2 antiserum in the gel diffusion tests. They did not produce coccus forms to any notable extent and fermented starch and raffinose and could therefore be distinguished from *Rothia dentocariosa* (Gerencser and Slack 1969). The pattern of the cell wall components was in accord with that described for *A. viscosus* (Bowden and Hardie 1973 a) and the reactions in the physiological tests were in line with previous observations (Gerencser and Slack 1969). The 3 isolates were identified as *A. viscosus*.

Two isolates had a pattern of cell wall components almost identical with that of *A. naeslundii* but neither of them had the biochemical characteristics of the reference strains or reacted in gel diffusion tests with the antisera. These isolates differed from all accepted species and were only provisionally diagnosed as *Actinomyces spp.*

Group 2

Two isolates were found to have an unusual pleomorphic cell morphology ranging from that of cocci to that of rods. They were catalase positive and grew best aerobically. Their growth was not stimulated by CO₂ (Chapter 2). Components of the cell wall were lysine and galactose but not ornithine (Table 3: 3). Both isolates reduced nitrite and hydrolysed gelatine but not starch. In all these respects the microorganisms resembled *Rothia* (Georg and Brown 1967, Brown *et al.* 1969, Schleifer and Kandler 1972, Leshner *et al.* 1974). Other characteristics made it possible to identify one of these isolates as *Rothia dentocariosa*. As for the other isolate lactic acid could not be detected after glucose fermentation and rhamnose was detected among the cell wall components. These characteristics are differed from those of *Rothia dentocariosa* (Georg

Table 3:3. *Cell wall components and biochemical characteristics of Rothia-like isolates and of reference strain.*

Characteristics	Characteristics of reference strain and isolates resembling the species	
	<i>Rothia dentocariosa</i>	
	ATCC 17931	isolates n = 2 ¹
Cell wall components		
galactose	+ ²	2 ³
glucose	tr	1
mannose	—	1
rhamnose	—	1
6-deoxytalose	—	0
Catalase	+	2
Hydrolysis of		
soluble starch	—	0
esculin	+	2
Reduction of		
nitrate	+	2
nitrite	+	2
Acid from		
arabinose	—	0
cellobiose	—	0
lactose	—	0
maltose	+	2
mannitol	—	0
mannose	—	2
raffinose	—	0
sucrose	+	2
xylose	—	0

Symbols

¹, ², and ³ see Table 3:2.

and Brown 1967). This isolate was therefore only tentatively considered as *Rothia* sp.

Group 3

Fourteen isolates were assigned to group 3 (Table 3:4) because they produced acetic and non-volatile acids in roughly equal amounts and because of their negative catalase reaction.

Ten of these isolates appeared with pleomorphic branching cell morphology. Bifid forms were observed mainly in wet preparations and

especially after culture on tomato agar (Sundman and af Björkstén 1958). These isolates were therefore regarded as *Bifidobacterium* (Sundman *et al.* 1959, de Vries *et al.* 1967, Pine 1970, Holdeman and Moore 1972, Poupard *et al.* 1973). The presence of lysine and ornithine as well as the carbohydrate components in the cell walls was in agreement with previous observations in *Bifidobacterium* (Schleifer and Kandler 1972) and *Lactobacillus* (*Bifidobacterium*) *bifidus* (Cummins *et al.* 1957) but not in *Bifidobacterium* (*Actinomyces*) *eriksonii* in which galactose and fucose can be demonstrated (Georg *et al.* 1965). The biochemical characteristics were almost identical with those of the reference strain *Bifidobacterium eriksonii* ATCC 15423. These isolates resembled *Bifidobacterium* group 4 according to Dehnert (1957) and Werner (1966) and the recently described species *Bifidobacterium dentium* sp. nov. isolated from dental caries (Scardovi and Crociani 1974). No reference strains of *Bifidobacterium* group 4 (Dehnert) or *B. dentium* sp. nov. were available for detailed examination. Because of their resemblance to the reference strain *Bifidobacterium eriksonii* ATCC 15423 in the biochemical tests, the ten isolates were regarded as members of this species.

In four isolates the cells differed morphologically from those in the other isolates in group 3. The configuration of the cell varied and although coccus-like cells predominated, pleomorphic and sometimes branching rods were observed. In wet preparations from cultures on tomato agar no distinct bifid cells were observed. All four isolates grew only anaerobically and gave only scanty growth in the standard media. They grew somewhat better in PRAS-media (Holdeman and Moore 1972) and in thioglycollate medium with glucose and serum. Although the fermentation pattern of these four isolates were similar in various respects to the reference strain *Bifidobacterium longum* ATCC 15707, the cells of the isolates differed from the latter in morphology and growth. These isolates were therefore only tentatively regarded as *Bifidobacterium* spp. (de Vries *et al.* 1967, Pine 1970, Holdeman and Moore 1972).

Group 4

All isolates in this group were Gram positive branching rods detected mainly in wet preparations. Their major acid end products were acetic and propionic acids. All were catalase negative. These characteristics suggest that the isolates might represent members of genus *Arachnia* (Gerencser and Slack 1967, Pine and Georg 1969, Holdeman and Moore 1972). In contrast to the cell wall of *A. propionica* the only

Table 3:4. *Cell wall components and biochemical characteristics of Bifidobacterium-like isolates and of reference strains.*

Characteristics	Characteristics of reference strains and isolates resembling respective species			
	Bifidobacterium/ Actinomyces eriksonii		Bifidobacterium dentium sp. nov.	Bifido- bacterium spp.
	ATCC 15423	isolates n = 10 ¹	Data from Scardovi and Crociani (1974) ATCC 27534	isolates n = 4 ¹
Cell wall components				
galactose	+	10 ³	ND	2/2 ⁴
mannose	—	7	ND	0/2
rhamnose	—	7	ND	0/2
Hydrolysis of				
esculin	+	9	ND	3
Gas from glucose	+	5	ND	0
Acid from				
arabinose	—	9	+	0
cellobiose	+	9	+	0
inulin	—	0	—	0
mannitol	+	10	+	0
mannose	+	10	+	0
melezitose	—	9	+	3
salicin	+	9	+	3
sorbitol	—	0	+	0
starch insoluble	+	10	ND	2
trehalose	+	8	+	3
xylose	+	9	+	3

Symbols

¹, ², ³, and tr see Table 3:2. ND no data available.

⁴ Results of cell wall analysis are available from only 2 of these isolates. Yield of growth from the other 2 isolates was not considered enough for the performance of this analysis. All the isolates had lysine and ornithine in their cell walls. No glucose, 6-deoxytalose, DL.DAP or LL.DAP was demonstrable. All fermented fructose, maltose, sucrose, glycogen and soluble starch; none fermented arabinose, rhamnose, inulin, esculin, erythritol, glycerol, inositol and sorbitol; all hydrolysed soluble and insoluble starch; none split gelatine or tributyrin; none produced H₂S and indol or reduced nitrate; no cytochrome system was demonstrable.

species in this genus, which contains LL.DAP, galactose and glucose (Johnson and Cummins 1972), all eleven isolates had DL.DAP together with galactose and glucose (Table 3:5). Comparison between the results in the biochemical tests for *A. propionica* ATCC 14157 and the isolates also revealed that they varied from the reference strain in several

Table 3: 5. *Some biochemical characteristics of Arachnia-like isolates and of the reference strain.*

Characteristics	Characteristics of the reference strains and isolates	
	<i>A. propionica</i>	
	ATCC 14157	isolates n = 11 ¹
Cell wall components		
DL.DAP	— ²	11 ³
LL.DAP	+	0
glucose	—	11
Hydrolysis of		
soluble starch ⁴	—	9v
Reduction of nitrate	+	5
Detection of cytochrome containing system	—	5
Acid from		
arabinose	+	0
erythritol	—	5
inositol	—	11
lactose	+	6
maltose	+	11
mannitol	+	10
salicin	—	0
sorbitol	+	0
sucrose	+	10
trehalose	+	1
xylose	+	0

Symbols

¹, ², ³, and V see Table 3: 2.

⁴ Similar pattern for hydrolysis of insoluble starch.

The cell wall of all the strains contained galactose. They all produced gas from glucose. None produced catalase and H₂S, hydrolysed esculin or split gelatine and tributyrin.

of the characteristics studied. Judging from these results the isolates in this group do not possess the typical characteristics of *A. propionica*. However, on the basis of their cell morphology, catalase reaction and acid end products all were identified as *Arachnia spp.*

Group 5

Ten isolates were Gram positive pleomorphic and sometimes branching rods. They were catalase positive and produced propionic acid as a

Table 3:6. *Cell wall components and biochemical characteristics of Propionibacterium-like isolates and of reference strains.*

Characteristics	Propionibacterium acnes		Propionibacterium avidum	Propionibacterium granulosum	Propionibacteriaceae
	NCTC 737	isolates: 9 ¹	ATCC 25577	Data from Cummins and Johnson 1974	isolate: 1 ¹
Cell wall components					
galactose	+ ²	9 ³	ND	+	1
glucose	+	9	ND	tr	1
mannose	tr	9 ^{tr}	ND	+	1 ^{tr}
Hydrolysis of					
esculin	—	0	+	—	0
Splitting of					
gelatine	+	9	+	— W	0
Reduction of					
nitrate	+	9	—	—	0
Production of					
indol	+	7	—	— or W	0
Fermentation of					
glucose	+	9	+	+	0
maltose	+	9	+	+	0
melezitose	—	0	+	V	0
sorbitol	+	6	—	—	0
sucrose	—	0	+	+	0

Symbols

¹, ², ³, tr and V see Table 3:2.

W Weak positive reaction or weak acid production (pH 5.5—6.0).

All the isolates had LL.DAP as a cell wall component, split tributyrin and had a demonstrable cytochrome system.

major end product. Cell wall components were LL.DAP, galactose, glucose and trace of mannose (Table 3:6). These characteristics taken together indicate that the isolates are members of *Propionibacteriaceae* (Holdeman and Moore 1972, Johnson and Cummins 1972, Moore and Holdeman 1973). Nine isolates did not grow on blood agar aerobically except when a heavy inoculum was used. The reactions to tributyrin, gelatine and their fermentation of carbohydrates showed that they resembled *Propionibacterium acnes* (Puhvel 1968, Johnson and Cummins 1972, Moore and Holdeman 1973, Cummins and Johnson 1974).

One isolate shared the basic characteristics of the nine isolates described above, but differed from them in being facultatively anaerobic and almost non-saccharolytic. This isolate was therefore only provi-

sionally regarded as a Gram positive facultative anaerobic rod within *Propionibacteriaceae* (Schleifer and Kandler 1972, Johnson and Cummins 1972, Moore and Holdeman 1973).

Group 6

Twelve strains formed a distinct group in that they produced a wide range of volatile acids including significant amounts of caproic acid. Non-volatile acids were not detected. They were pleomorphic rods but branching was not apparent. All produced gas from glucose. They grew only anaerobically on blood agar. All were catalase negative. Taken together the results suggest that they may be members of *Eubacterium* (Holdeman and Moore 1972, Moore and Holdeman 1973).

All the twelve isolates were shown to have DL.DAP, galactose, glucose, rhamnose, but not lysine, ornithine, mannose or 6-deoxytalose as cell wall components. Only limited data are available about cell wall components in the genus *Eubacterium*. Holdeman and Moore (1972) reported DL.DAP to be present in some of the species including *E. alactolyticum*. To our knowledge nothing has been reported about the carbohydrate components (Table 3: 7).

In the biochemical tests all the strains behaved most like the reference strains *Eubacterium alactolyticum* ATCC 19301 and *Eubacterium limosum* ATCC 8486. Because of the presence of DL.DAP in the cell walls, the production of caproic acid and their inability to split esculin all twelve isolates could be distinguished from *E. limosum* and were identified as *E. alactolyticum* (Holdeman and Moore 1972, Moore and Holdeman 1973, Holdeman 1974 personal communication).

Remarks

The assignment of the isolates to genera was based mainly on the results in the acid end product analysis. Without such data allocation of any isolate to a certain genus might have been difficult.

Most *Actinomyces*-like isolates could be assigned to accepted species mainly on the basis of gel diffusion- and cell wall-analyses. But it is clear that some strains were not typical of accepted species. In fluorescent antibody studies of *Actinomyces* isolates it is known that some strains do not stain typically (Brock and Georg 1969, Slack *et al.* 1969). It seems possible that these variations could be related in part to differences in cell wall carbohydrates. Similar differences might explain the variation observed in the present gel diffusion analysis.

The groups of *Actinomyces spp.* obtained did not show any distinct

Table 3: 7. *Cell wall components and physiological characteristics of Eubacterium-like isolates and of reference strains.*

Characteristics	Characteristics of reference strains and isolates resembling respective species		
	E. alactolyticum		E. limosum
	ATCC 19301	isolates subgroups n = 12 ¹	ATCC 8486
Cell wall components			
DL.DAP	+ ²	12 ³	—
LL.DAP	—	—	—
galactose	+	12	tr
glucose	+	12	+
rhamnose	+	12	+
Hydrolysis of			
esculin	—	0	+
Acid from			
amygdalin	—	0	—
erythritol	—	4W	+
fructose	+	12	+
glycogen	—	7W	—
lactose	—	2	—
maltose	—	2W	—
mannitol	W	7W	+
mannose	—	9W	—
raffinose	—	4W	—
rhamnose	—	0	—
starch	—	0	—
sucrose	—	1W	—
trehalose	—	1W	—
xylose	—	0	—

Symbols

¹, ² and ³ see Table 3: 2.

W Indicates weak acid (pH 5.5—6.0) and/or variable results.

Lysine, ornithine, mannose or 6-deoxytalose were not detected in cell wall preparations from any of the isolates. All isolates produced gas from glucose, none produced indol or reduced nitrate.

pattern of biochemical characteristics. Variations of these characteristics are known to occur among members of *Actinomyces* and may be dependent on the choice of media, anaerobiosis etc. (Slack *et al.* 1968, Gerencser and Slack 1969). On the other hand, there does not seem to be any single test which differentiates species within the *Actinomyces* (Bowden and Hardie 1973 a).

Perhaps the easiest group to characterize biochemically was the one that resembled the anaerobic *Propionibacterium* where the inability to grow aerobically associated with a strong catalase positive reaction and splitting of tributyrin gave a fairly good indication of the character of the isolate.

Cell wall analysis was useful in corroborating the identification in several of the groups. It should be emphasized, however, that some of the isolates were more or less provisionally identified and grouped. A study of a wider spectrum of characteristics might have led to a better identification. But it has recently been reported that, despite the use of more than 500 characteristics, oral Gram positive rods were still difficult to identify accurately (Spellman *et al.* 1974).

SUMMARY

Sixty-four isolates being tentatively identified as Gram positive pleomorphic rods were further studied with gas chromatographic analysis of acid end products after glucose fermentation, cell wall analysis, gel diffusion analysis and biochemical methods. The isolates could be divided into 6 groups corresponding to the genera *Actinomyces*, *Rothia*, *Bifidobacterium*, *Arachnia*, *Propionibacterium* and *Eubacterium*. Fourteen isolates resembling *Actinomyces israelii*, *A. naeslundii*, *A. odontolyticus* and *A. viscosus* were assigned to group 1. Two of the isolates in this group were only provisionally identified as *Actinomyces spp.* Group 2 consisted of 2 isolates resembling *Rothia spp.* Group 3 constituted 14 isolates identified as *Bifidobacterium spp.*; ten of these isolates resembled *Bifidobacterium (Actinomyces) eriksonii* in many aspects but also the recently described *Bifidobacterium dentium* sp. nov. Another 4 isolates in this group shared only some basic characteristics with those of *Bifidobacterium* and were therefore provisionally identified as *Bifidobacterium spp.* Group 4 consisted of 11 isolates which, judging from the chromatographic analysis and catalase reaction, were *Arachnia spp.* They differed, however, from the only species within this genus, *Arachnia propionica* in the composition of the cell wall and the pattern of reactions in the biochemical tests and were therefore only provisionally identified as *Arachnia spp.* Group 5 consisted of 10 isolates with a fairly distinct pattern of characteristics which suggested that the majority of them were *Propionibacterium acnes*. Twelve isolates were assigned to Group 6 and on the basis of a broad pattern of acid end products were identified as *Eubacterium* and further assessed as *E. alactolyticum* from their composition of cell walls and their biochemical behaviour.

4. Identification of *Lactobacillus*-like isolates

Ninety-six isolates were Gram positive rods. All were catalase-negative facultative anaerobic, non-motile and aciduric judged by their excellent growth in Rogosa SL-agar (Rogosa *et al.* 1951). The majority of them fermented glucose in O-F- and CTA-medium, some with gas production, none of them reduced nitrate. They may therefore be considered as members of genus *Lactobacillus* (Rogosa *et al.* 1951, Rogosa *et al.* 1953, Breed *et al.* 1957, Cowan and Steel 1965). Eighty-four of these isolates were studied more closely together with certain reference strains within genus *Lactobacillus* in order to identify them.

MATERIAL AND METHODS

All eighty-four isolates were collected from the blood agar plates incubated 2 or 7 days anaerobically (2H or 7H), or from the TA-Ser medium used at the primary incubation of dentine material.

All the isolates were stored in the freeze-dried state before the investigation. During the study the strains were maintained on blood agar at +6°C and subcultured each 3rd week. The inoculum for all the tests consisted of an 18 hour broth culture (Bacto Lactobacilli MRS Broth, Difco 0881). The culture was washed twice in 0.02 M Naphosphate buffer, pH 6.6, containing 0.4 per cent (w/v) NaCl. Unless otherwise stated the inoculum for the broth media tests consisted of 0.1 ml of the washed culture.

The viable-count per drop of the inoculum broth was determined on blood agar plates incubated 2 days in 95 % N₂ and 5 % CO₂ after dilution in the pH 6.6-buffer solution. If the count was less than 10⁴ all the inoculated test media were discarded.

All the tests were run twice. Where the results did not agree, a third test was run. The final result of the test was recorded as that which occurred twice in three tests.

All the aseptic procedures at the media preparation were made in a Laminar Flow Work Station (Favero and Berquist 1968). All the media

were subjected to an incubation at $+37^{\circ}\text{C}$ for 3 days in order to detect any contaminating microorganisms capable of growing at that temperature. The base medium followed the formula for Lactobacilli MRS Broth given by Difco (code 0881) with the preparations as described below.

Solution I (subsequently called Sol I) consisted of Tryptone (Difco 0123-02) to a final concentration in the medium of 2 % (w/v), Yeast Extract (Difco 0127-01), final concentration 1 % (w/v), Tween 80, final concentration 0.1 % (w/v) and distilled water. Sol I was autoclaved at 120°C for 15 min. in 3 or 5 ml amounts in screw-capped test tubes.

Solution II (subsequently called Sol II) consisted of the salts ammonium citrate, sodium acetate, magnesium sulfate and dipotassium phosphate in distilled water added to a final concentration in medium as described for Lactobacilli MRS Broth (Difco code 0881). Sol II was always sterilized by filtration (Gelman Instrument Co., Ann Arbor, Mich. Metrical Membrane Filter, GA-8, 0.2 micrometer).

Fermentation tests. Sol I in double strength was distributed in screw-capped test tubes with 2.5 ml per tube and autoclaved at $+120^{\circ}\text{C}$ for 15 minutes. The carbohydrates used are given in Table 4:4. A ten per cent (w/v) solution of each carbohydrate was prepared with distilled water. The following carbohydrate solutions were autoclaved at $+115^{\circ}\text{C}$ for 15 minutes: alpha-methyl-D-glucoside, amygdalin, cellobiose, fructose, glycerol, glycogen, inositol, lactose, mannitol, melibiose, melezitose, raffinose, rhamnose, salicin, soluble starch, sorbitol, sucrose, trehalose. The other carbohydrate solutions were sterilized by filtration (Metricel, Membrane Filter GA-8) according to the recommendation of Rogosa and Sharpe (1959).

The carbohydrate solutions were mixed with an appropriate amount of Sol II and the mixture added to the test tubes with Sol I to a final volume in the tubes of 5 ml. The pH in the final sterilized medium was 6.5–6.7. One drop (approx. 0.05 ml) of the strain at test was inoculated into each tube.

Turbidity was noted by eye and the pH was read with a pH-meter after 14 days incubation. A pH lower than 5.4 was considered as a positive reaction (Hansen *et al.* 1968) and between 5.4 to 5.7 a weak positive reaction.

For the determination of growth at $+15^{\circ}\text{C}$ and $+45^{\circ}\text{C}$ Lactobacilli MRS Broth (Difco code 0881) was dispensed in screw-capped tubes in amounts of 5 ml and autoclaved at $+120^{\circ}\text{C}$ for 15 minutes. The tubes were placed in the water baths a couple of hours before the incubation. The pH and the turbidity were read after 7 days. Turbidity and a drop in pH to less than 5.4 was considered a positive reaction, between 5.4 and 5.7 as a weak positive reaction.

Production of NH_3 from arginine. The base medium for fermentation tests (Sol I + Sol II) was supplemented with 0.3 % (w/v) L-arginine hydrochloride and 2 % (w/v) glucose as recommended by Briggs (1953). Arginine and glucose were dissolved in 500 ml distilled water, autoclaved at $+120^\circ\text{C}$ for 30 minutes and mixed with Sol II. This mixture was distributed into tubes containing Sol I to a final volume of 8 ml per tube.

Hydrolysis of sodium hippurate. Sol I + Sol II were supplemented with glucose 0.5 % (w/v) and sodium hippurate 1.0 % (w/v). The same procedures as described for arginine were carried out. Incubation and reading were performed according to Davis (1955).

Production of gas from glucose and gluconate. Sol I + Sol II were supplemented with beef extract (Difco 0126-01) to give a final concentration in the medium of 1 % (w/v), and agar (Difco Bacto-Agar 0410-01) to a final concentration of 2 % (w/v). The glucose medium contained 2 % (w/v) glucose as recommended by Gibson and Abdel-Malek (1945). The gluconate medium contained 4 % (w/v) sodium gluconate (British Drug House) and the pH was adjusted to 5.2 (Rogosa 1971 a).

The agar and glucose or gluconate were dissolved in 500 ml distilled water, dispensed into screw-capped tubes with 5 ml in each and autoclaved at $+115^\circ\text{C}$ for 15 minutes.

The recommendation to use large inocula for these tests (Gibson and Abdel-Malek 1945, Rogosa *et al.* 1953) was followed. Five ml volumes per tube of Sol I + Sol II were inoculated with 0.5 ml of the strain under test. The agar-carbohydrate-solutions were melted, placed in a water bath at $+45^\circ\text{C}$ for 30 minutes, mixed evenly with the inoculated Sol I + Sol II and put in upright position. The tubes were sealed, incubated (14 days) and read according to Rogosa *et al.* (1953).

Growth in 0.2 %, 0.3 % and 0.4 % Teepol were studied with the media and methods described by Hansen *et al.* (1968). pH in the final broth was 6.0. Turbidity and a pH lower than 5.1 was considered as a positive reaction.

Fermentation products from glucose were studied by a gas chromatographic procedure described by Carlsson (1973). All the reference strains and isolates were maintained on blood agar plates and from these plates inoculated into the modified PRAS-broth (Carlsson 1973), incubated at $+37^\circ\text{C}$ until good growth appeared (usually after 2—3 days). The test tubes were immediately sent from Malmö to Umeå where the analysis of fermentation products was done by Dr. J. Carlsson at the Department of Oral Microbiology, University of Umeå.

At the determinations of L- and D-lactic acid all the reference strains and isolates within *Thermobacterium* were cultured in MRS-broth with-

Table 4: 1. *Reference strains and their sources.*

Lactobacillus acidophilus	NCTC 1
Lactobacillus brevis	NCDO 477
Lactobacillus buchneri	NCDO 110
Lactobacillus casei var. casei	NCTC 151
Lactobacillus casei var. rhamnosus	ATCC 7469
Lactobacillus cellobiosus	NCDO 928
Lactobacillus fermentum	NCTC 1750
Lactobacillus jensenii	CIP 6917
Lactobacillus jugurti	NCDO 87
Lactobacillus lactis	NCDO 1438
Lactobacillus leichmanni	NCIB 7854
Lactobacillus plantarum var. arabinosus	NCDO 82
Lactobacillus plantarum	NCDO 343
Lactobacillus salivarius var. salicinius	NCDO 929

out meat extract for 48 hours and were immediately sent to Dr. J. Carlsson, Department of Oral Microbiology, University of Umeå. Total lactic acid was determined as described by Carlsson (1973) and L-lactic acid as described by Hohorst (1970).

For analysis of amino acid composition of the cell wall, the isolates were cultured for 3 days in 1.5 litre MRS-broth with 2 % glucose (w/v) following the formula given above. The cultures were centrifuged at $8000 \times g$ at $+4^{\circ}\text{C}$ for 15 minutes. The supernatant was discharged and the pellet was lyophilized. Depending on the growth intensity in the different cultures, the final weight of the lyophilized material ranged from 1.5 to 3.1 grams. The lyophilized material was sent to Dr. R. A. D. Williams, Department of Biochemistry, The London Hospital Medical College, London, who prepared and examined the cell walls according to the method described by Williams and Sadler (1971) and Williams (1971).

The reference strains are given in Table 4: 1.

RESULTS AND DISCUSSION

Based on the observation of lactic acid as major acid end product and on the preliminary data presented in chapter 2 for group II, all the isolates could be considered as belonging to genus *Lactobacillus* (Breed *et al.* 1957, Rogosa and Sharpe 1959, Moore and Holdeman 1973). Thus, the characteristics used in Chapter 2 to bring certain isolates into group II were of differential value.

Production of only lactic acid placed some of the isolates within *Thermobacterium*/*Streptobacterium* while production of lactic acid, acetic acid and ethanol placed the other isolates into *Betabacterium* (Breed *et al.* 1957, Rogosa and Sharpe 1959).

The homofermentative organisms were divisible into *Thermobacterium* and *Streptobacterium* on the basis of gas production from gluconate and growth at temperatures of +15°C and +45°C (Table 4:2). Rogosa (1970) reported that *Thermobacterium* could be separated from *Streptobacterium* since the members of the latter subgenus, in contrast to those of the former, produced acid from ribose. In the present study it was observed that *L. jensenii*, a new representative of *Thermobacterium* (Gasser *et al.* 1970) in repeated tests fermented ribose. Rogosa (1970) did not include *L. jensenii* in his report and Gasser *et al.* (1970, 1973 personal communication) have for the moment not tested *L. jensenii* for ribose fermentation. However, ribose fermenting strains resembling *L. jensenii* have been isolated from the recto-vaginal region of mothers and in the mouth of the children at the time of delivery (Carlsson and Gothefors 1973, personal communication).

Of the twenty-two isolates within subgenus *Betabacterium*, fifteen deaminated arginine. Variations were also observed in this group concerning hydrolysis of esculin and hippurate, gas production from gluconate, acid production from ribose as well as growth at +15°C and +45°C. Similar variations have previously been reported (Davis 1955, Rogosa and Sharpe 1959, Rogosa 1970).

The nutrition of lactobacilli is considered to be complex and it is necessary to use enriched substrates such as MRS-medium in order to minimize variations in the test results (Rogosa *et al.* 1953). Under adequate conditions, however, individual strains of lactobacilli have proved remarkably consistent in their characteristics (Rogosa and Sharpe 1959). Despite the use of MRS-broth as base medium in the present study, the results of certain key characteristics for some of the reference strains were found to be different from those reported previously (Table 4:3). The observed results were consistent when the tests were repeated twice with an inoculum containing more than 10^5 viable cells (per drop) and when freshly prepared media were used. Neither was any obvious change observed when the tests were repeated in PRAS-medium (Holdeman and Moore 1972). When the isolates were listed into species (Tables 4:4—7), the results of the test on those characteristics were not considered as “key”-characteristics since they differed from the results presented by several investigators.

Table 4: 2. Some important differentiating characteristics of *Lactobacillus*.

	Gas production:		Acid from ribose	Deamination of arginine	Hydrolyse of:		Growth at:	
	glucose	gluconate			esculin	hippurate	+ 15°C	+ 45°C
<i>Thermobacterium</i>								
<i>L. acidophilus</i> NCTC 1	—	—	—	—	+	+	—	+
<i>L. jensenii</i> CIP 6917	—	—	+	+	+	+	—	+
<i>L. lactis</i> NCDO 1438	—	—	—	—	+	—	—	+
<i>L. salivarius</i> var. <i>salicinius</i> NCDO 929	—	—	—	—	+	—	—	+
<i>L. leichmanni</i> NCIB 7854	—	—	—	+	+	—	—	+
<i>L. jugurti</i> NCDO 87	—	—	—	—	—	—	—	+
Isolates:	0/22*	0/22	3/22	1/22	21/22	0/22	3/22	22/22
<i>Streptobacterium</i>								
<i>L. casei</i> var. <i>casei</i> NCTC 151	—	+	+	—	+	+	+	±
<i>L. casei</i> var. <i>rhamnosus</i> ATCC 7469	—	+	+	—	+	+	+	+
<i>L. plantarum</i> NCDO 343	—	+	+	—	+	+	+	—
<i>L. plantarum</i> var. <i>arabinosus</i> NCDO 82	—	+	+	—	+	+	+	—
Isolates:	0/40	40/40	40/40	0/40	40/40	38/40	38/40	38/40
<i>Betabacterium</i>								
<i>L. fermentum</i> NCTC 1750	+	+	+	+	—	—	—	+
<i>L. buchneri</i> NCDO 110	+	+	+	+	—	+	+	—
<i>L. brevis</i> NCDO 477	+	+	+	+	+	+	+	—
<i>L. cellobiosus</i> NCDO 928	+	+	+	+	+	—	+	+
Isolates:	22/22	16/22	16/22	15/22	9/22	11/22	6/22	20/22

Symbols

+ = positive reaction, — = negative reaction.

* Number of positive isolates/Number of isolates assigned to respective subgenera.

Table 4: 3. *Observed differences among the reference strains concerning certain key characteristics.*

Reference strains	Reaction/ Fermen- tation of	Results			
		Rogosa <i>et al</i> 1953 Rogosa and Sharpe 1959	Hansen <i>et al</i> 1968	Own MRS medium	PRAS medium
<i>L. brevis</i> NCDO 477	arabinose	+	+	-	-
<i>L. buchneri</i> NCDO 110	arabinose	+	+	-	-
	melezitose	+	+	-	-
<i>L. lactis</i> NCDO 1438	galactose	+	+	-	+
	cellobiose	-	+	+	+
	esculin				
<i>L. plantarum</i> var. <i>arabinosus</i> NCDO 82	(hydrolysis of) arabinose	- + or -*	+ or - +	+ -	nt** W**

Symbols

See Table 4: 2.

* About one half of the strains fermented the sugar.

** W = weak fermentation; nt = not tested.

Isolates identified as Thermobacterium

Twenty-two isolates were allocated to *Thermobacterium* (Table 4:4). Twenty-one of these isolates formed DL-lactic acid, hydrolysed esculin, grew at +45°C, fermented cellobiose, sucrose, trehalose and maltose. A majority of them also fermented amygdalin, fructose, galactose, lactose, mannose, salicin and grew in the presence of 0.2 per cent Teepol. They share many of these characteristics with the reference strain *L. acidophilus*. They could be separated from *L. jensenii*, *L. lactis* and *L. leichmanni* as these strains form D-lactic acid, and from *L. jugurti* NCDO 87, which also produces DL-lactic acid, by differences in the fermentation of various carbohydrates. The production of DL-lactic acid and the pattern of carbohydrate fermentation brought these twenty-one isolates into the species *L. acidophilus* (Rogosa and Sharpe 1959, Gasser 1970, Hansen and Mocquot 1970).

One of the isolates resembled *L. salivarius* var. *salicinicus*. It produced L-lactic acid and fermented mannitol, salicin and sorbitol. These characteristics together with the failure to ferment amygdalin and cellobiose separated it from *L. acidophilus*.

Isolates identified as Streptobacterium

Forty isolates were placed within *Streptobacterium*. These isolates could be divided into 2 groups (Table 4:5).

One group consisted of 2 isolates which produced DL-lactic acid. One of them had diaminopimelic acid (DAP) in the cell wall. In most characteristics they were similar to the reference strains *L. plantarum* NCDO 343 and *L. plantarum* var. *arabinosus* NCDO 82 and they were therefore considered as members of the species *L. plantarum* (Rogosa and Sharpe 1959, Hansen *et al.* 1968, Kandler 1970, Williams 1971).

The other group consisted of 38 isolates, which differed from the isolates in the above mentioned group in several characteristics. The pattern of biochemical characteristics were similar to those of the reference strains of *L. casei*. Preliminary tests of total lactic acid production in MRS-broth with glucose (method according to Barker and Summerson [1941]) and the production of L-lactic acid (method according to Hohorst 1970) indicate that the members in this group, in contrast to those resembling *L. plantarum*, only produced L-lactic acid. They were therefore identified as *L. casei* (Rogosa and Sharpe 1959, Sharpe 1962, Hansen and Lessel 1971). Based on the ability to ferment rhamnose, twenty of them were placed in the subspecies *L. casei* var. *rhamnosus* (Rogosa and Sharpe 1959, Hansen *et al.* 1968).

Table 4: 4. *Characteristics of isolates assigned to subgenus Thermobacterium.*

Characteristics	L. acidophilus		L. lactis	L. leichmanni	L. jensenii	L. salivarius		L. jugurti
	NCTC 1 isolates n = 21 ²	DL	NCDO 1438	NCDO 7854	CIP 6917	NCDO 929	isolate n = 1	NCDO 87
Type of lactic acid produced ¹	DL	DL	D (-)	D (-)	D (-)	L (+)	L (+)	DL
Gas from glucose	- ⁴	0 ³	-	-	-	-	0	-
gluconate	-	0	-	-	-	-	0	-
Deamination of arginine	-	1	-	+	+	-	0	-
Hydrolysis of esculin	+	21	+	+	+	+	0	-
Hydrolysis of hippurate	+	0	-	-	+	-	0	-
Growth at +15°C	-	3	-	-	-	-	0	-
Growth at +45°C	+	21	+	+	+	+	1	+
Fermentation of								
alpha-methyl-D-glucoside	-	0	-	-	+	-	0	-
amygdalin	+	20	+	+	+	-	0	-
arabinose	-	0	-	-	-	-	0	-
cellobiose	+	21	+	+	+	-	0	-
fructose	+	21	+	+	+	+	1	-
galactose	+	20	-	-	+	+	1	+
glycerol	-	0	-	-	-	-	0	-
glycogen	-	0	-	-	-	-	0	-
inositol	-	0	-	-	-	-	0	-
inulin	+	0	-	-	-	-	0	-
lactose	+	15	+	+	-	+	1	+
maltose	+	21	+	+	+	+	1	-
mannitol	-	0	-	-	-	+	1	-
mannose	+	21	-	+	+	+	1	+
melezitose	-	0	-	-	-	-	0	-

melibiose	+	1	-	-	-	+	1	-
raffinose	+	3	±	-	-	+	1	-
rhamnose	-	0	-	-	-	-	0	-
ribose	-	3	-	-	+	-	0	-
salicin	+	19	+	+	+	+	0	-
sorbitol	-	0	-	-	-	+	1	-
sorbose	-	0	-	-	-	-	0	-
starch	-	0	-	-	-	-	0	-
sucrose	+	21	+	+	+	+	1	-
trehalose	+	20	+	+	+	+	1	-
xylose	-	0	-	-	-	-	0	-
Growth in Teepol								
0.2 %	+	17	+	+	+	+	1	-
0.3 %	-	0	-	-	-	-	1	-
0.4 %	-	0	-	-	-	-	0	-

Results of all the biochemical tests for reference strains and isolates.

¹ The determination of the lactic acid isomers was made by Dr. J. Carlsson, Department of Oral Microbiology, University of Umeå.

² Number of isolates assigned to respective species.

³ Number of isolates positive in respective tests.

⁴ + = positive result or pH lower than 5.4; ± = pH 5.4—5.7; - = negative result or pH higher than 5.7.

Table 4: 5. *Characteristics of isolates assigned to subgenus Streptobacterium.*

Characteristics	L. casei var. rhamnosus		L. casei var. casei		L. plantarum		L. planta- rum var. arabinosus
	ATCC 7469	isolates n=20 ²	NCTC 151	isolates n=18	NCDO 343	isolates n=2 ⁴	NCDO 82
Gas from							
glucose	— ¹	0	—	0	—	0	—
gluconate	+	20	+	18	+	2	+
Deamination of arginine	—	0	—	0	—	0	—
Hydrolysis of esculin	+	20	+	18	+	2	+
Hydrolysis of hippurate	+	20	+	18	+	0	+
Growth at +15°C	+	20	+	18	+	0	+
Growth at +45°C	+	20	±	16	—	2	—
Fermentation of							
alpha-methyl-D-							
glucoside	+	15	+	16	—	0	+
amygdalin	+	20	+	18	+	2	+
arabinose	—	3	—	7	—	0	—
cellobiose	+	20	+	18	+	2	+
fructose	+	20	+	18	+	2	+
galactose	+	20	+	18	+	2	+
glycerol	—	2	—	8	—	0	—
glycogen	—	1	±	2	—	0	—
inositol	+	11	—	17	—	0	—
inulin	—	1	—	8	—	0	—
lactose	+	20	+	18	+	2	+
maltose	+	20	+	18	+	2	+
mannitol	+	20	+	18	+	0	+
mannose	+	20	±	18	+	2	+
melezitose	+	20	+	16	+	0	+
melibiose	—	0	—	0	+	2	+
raffinose	—	2	—	0	+	2	+
rhamnose	+	20	—	0	—	0	+
ribose	+	20	+	18	+	1	+
salicin	+	20	+	18	+	2	+
sorbitol	+	20	—	18	+	0	+
sorbse	+	13	—	15	—	0	—
starch	—	0	±	2	—	0	—
sucrose	+	20	+	18	+	2	+
trehalose	+	20	+	18	+	2	+
xylose	—	0	—	0	—	0	—
Growth in Teepol							
0.2 %	+	20	+	18	+	2	+
0.3 %	—	20	—	15	+	1	+
0.4 %	—	17	—	12	+	0	+

Results of all the biochemical tests for reference strains and isolates.

¹ + = positive result or pH lower than 5.4; ± = pH 5.4—5.7; — = negative result or pH higher than 5.7.

Table 4: 6. *Cell wall amino acid composition of heterofermentative isolates (Williams 1973 personal communication).*

Isolates resembling	Number of isolates	Cell wall amino acids	
		ornithine	lysine
<i>L. fermentum</i>	7	+	—
<i>L. fermentum</i> Reuter type II	2	—	+
<i>L. cellobiosus</i>	2	+	—
<i>L. buchneri</i>	3	—	+
<i>L. brevis</i>	1	—	+
Isolates being arginine negative	7	—	+

Isolates classified as Betabacterium

Twenty-two isolates were brought into *Betabacterium* (Tables 4: 6—7). Based on the cell wall amino acids, they were divided into two groups. Nine isolates contained ornithine but no lysine. Thirteen isolates contained lysine but no ornithine. The presence of these amino acid patterns makes it possible to consider the ornithine containing organisms as *L. fermentum* or *L. cellobiosus* while those containing lysine may belong to any of the species *L. brevis*, *L. buchneri* or *L. viridescens* (Williams 1971, Williams and Sadler 1971, Schleifer and Kandler 1972) (Table 4: 6).

In the biochemical tests, the above grouping of the isolates mainly were verified. Those isolates brought into the group *L. fermentum/L. cellobiosus* were similar to the reference strains *L. fermentum* NCTC 1750 and *L. cellobiosus* NCDO 928 in many characteristics. Therefore all the ornithine containing isolates being non-cellobiose fermenters were considered as members of species *L. fermentum*, while the two isolates which fermented cellobiose were given the name *L. cellobiosus* (Rogosa and Sharpe 1959, Hansen *et al.* 1968, Williams 1971, Schleifer and Kandler 1972) (Table 4: 7).

It was also observed that a pattern of characteristics similar to *L. fermentum* NCTC 1750 characterized two of the isolates containing lysine. Recently a variety of *L. fermentum* with lysine but no ornithine

² Number of isolates assigned to the respective species.

³ Number of isolates positive in respective tests.

⁴ No diaminopimelic acid (DAP) was detected in their cell wall (Williams 1973, personal communication), but both isolates produced DL-lactic acid isomers (Carlsson 1973, personal communication).

Table 4: 7. *Characteristics of isolates assigned to subgenus Betabacterium.*

Characteristics	L. fermentum		L. cellobiosus		L. buchneri		L. brevis		Arginine negative isolates resembling (L. brevis) n = 7	L. viridescens Results by Lessels and Rogosa (1971)
	NCTC 1750	isolates n = 9 ²	NCDO 928	isolates n = 2	NCDO 110	isolates n = 3	NCDO 477	isolates n = 1		
Gas from										
glucose	+ ¹	9 ²	+	2	+	3	+	1	7	+
gluconate	+	3	+	2	+	3	+	1	7	nt ⁴
Deamination of arginine	+	9	+	2	+	3	+	1	0	—
Hydrolysis of esculin	—	0	+	1	—	0	+	1	7	—
Hydrolysis of hippurate	—	0	—	0	+	3	+	1	7	—
Growth at +15°C	—	0	+	1	+	3	+	1	1	+
Growth at +45°C	+	9	+	2	—	2	—	0	7	—
Fermentation of										
alpha-methyl-D-glucoside	—	4	±	0	+	0	+	1	6	—
amygdalin	—	0	—	0	—	0	—	0	7	—
arabinose	—	0	—	1	—	0	—	0	0	—
cellobiose	—	0	+	2	—	0	—	0	0	—
fructose	+	9	+	2	+	3	+	1	7	+
galactose	+	9	+	2	+	3	+	1	7	—
glycerol	—	0	—	0	—	0	—	0	0	—
glycogen	—	0	—	0	—	0	—	0	0	—
inositol	—	0	—	0	—	0	—	0	0	—
inulin	—	0	—	0	—	0	—	0	0	—
lactose	+	9	+	2	+	3	+	1	6	—
maltose	+	9	+	2	+	3	+	1	7	+
mannitol	—	0	—	0	—	0	—	1	2	—
mannose	—	8	+	2	—	0	—	0	0	+
melezitose	—	0	—	0	—	3	—	0	0	—
melibiose	+	9	+	2	+	3	+	1	7	—

raffinose	+	9	+	2	+	3	-	0	7	-
rhamnose	-	0	-	0	-	0	-	0	1	nt
ribose	+	4	+	2	+	3	+	1	6	nt
salicin	-	0	±	1	-	0	+	1	6	-
sorbitol	-	0	-	1	-	0	-	0	0	-
sorbose	-	0	-	0	-	0	-	0	0	-
starch	-	0	-	0	-	0	-	0	0	-
sucrose	+	9	+	2	+	3	-	0	7	+
trehalose	-	0	+	2	-	0	-	0	0	-
xylose	-	0	-	0	+	0	+	1	7	-
Growth in Teepol										
0.2 %	+	9	+	2	+	3	+	1	7	nt
0.3 %	+	8	+	2	+	3	+	1	7	nt
0.4 %	+	1	+	1	+	3	+	1	5	nt

Results of all the biochemical tests studied both for reference strains and isolates.

¹ + = positive result or pH lower than 5.4; ± = pH 5.4—5.7; - = negative result or pH higher than 5.7.

² Number of isolates assigned to the respective species.

³ Number of isolates positive in respective tests.

⁴ Lessel and Rogosa (1971) did not report any results of these tests.

in the cell wall has been observed, viz. *L. fermentum* Reuter type II (Schleifer and Kandler 1972). The present two isolates may be identical with this variety which is considered as a separate species not yet described (Schleifer and Kandler 1972).

Four lysine containing isolates showed similarities in several of their biochemical characteristics with the reference strains *L. buchneri* and *L. brevis*. Three of these isolates fermented melezitose, raffinose and sucrose and were therefore considered as members of species *L. buchneri* while the fourth isolate which did not ferment these carbohydrates was brought into the species *L. brevis* (Sharpe 1962, Hansen *et al.* 1968, Rogosa 1970).

Seven lysine containing isolates failed to deaminate arginine and in these aspects they are similar to *L. viridescens* (Rogosa and Sharpe 1959, Lessel and Rogosa 1971, Williams 1971, Schleifer and Kandler 1972). In contrast to these seven isolates, *L. viridescens* only ferments few carbohydrates (Lessel and Rogosa 1971) and the isolates were therefore considered to be more related to *L. brevis*/*L. buchneri*. The majority of them hydrolysed esculin, fermented salicin, xylose and alpha-methyl-D-glucoside but not melezitose. In these characteristics they were similar to *L. brevis* NCDO 477. In agreement with *L. buchneri* NCDO 110 on the other hand, all of them fermented raffinose and sucrose, characteristics observed to differ this species from *L. brevis* (Hansen *et al.* 1968). Thus, with the characteristics studied it was not possible to make a more definite identification. Based on the overall similarities the arginine negative isolates were tentatively placed in the species *L. brevis*.

Remarks

After the basic grouping into the subgenera *Thermobacterium*, *Streptobacterium* and *Betabacterium*, it was found of value for the further identification to study both the production of lactic acid isomers, amino acid composition of the cell walls and the different biochemical characteristics (pattern of carbohydrate fermentation, hydrolyse of hippurate and esculin etc.). Reliance on biochemical characteristics only, might have lead to a more uncertain identification.

Despite of the few variations found when comparing the present results for the reference strains in the "key"-characteristics with those given in previous studies (Rogosa and Sharpe 1959, Sharpe *et al.* 1966, Hansen *et al.* 1968, Rogosa 1970, Williams 1971, Schleifer and Kandler 1972), it was not possible to give a more definite identification for all the isolates. Seven arginine negative isolates shared "key"-characteristics

Table 4: 8. *Frequency distribution of lactobacilli.*

Resembling: subgenus/species	Isolates	
	No	Per cent approx.
<i>Thermobacterium</i>		
L. acidophilus	21	25
L. salivarius var. salicinius	1	1
<i>Streptobacterium</i>		
L. casei var. rhamnosus	20	24
L. casei var. casei	18	22
L. plantarum	2	2
<i>Betabacterium</i>		
L. fermentum	9	10
L. cellobiosus	2	2
L. buchneri	3	4
L. brevis	1	1
Arginine negative (L. brevis)	7	8

both with *L. brevis* and *L. buchneri*. A close relationship between these two species has recently been pointed out by Gasser and Mandel (1968) and by Williams (1971) and considering the variation that could occur depending on the basal medium, anaerobiosis etc. (Rogosa *et al.* 1953, Rogosa 1970), only a tentative identification was made.

SUMMARY

Eighty-four isolates which were preliminarily placed within group II (Chapter 2) could be identified as facultative anaerobic lactobacilli. The majority of isolates belonged to subgenus *Streptobacterium* (Table 4: 8) and closely resembled *L. casei* var. *rhamnosus* and var. *casei* (24 and 22 per cent of all the isolates respectively)—two isolates were considered as related to *L. plantarum*.

The two subgenera *Thermobacterium* and *Betabacterium* were represented in about equal amounts (26 and 25 per cent respectively of all isolates studied).

5. Identification of *Streptococcus*-like isolates

Twenty-eight isolates brought into identification group III, were Gram positive cocci which appeared in chains in stained films from broth and semisolid broth cultures. All these isolates were non-motile and catalase negative. All fermented glucose and grew well on Mitis-Salivarius agar and blood agar aerobically. They may be considered as belonging to family *Streptococcaceae* (Breed *et al.* 1957, Cowan and Steel 1965, Chapman 1944).

Nineteen of these isolates were examined more closely by a number of morphological and physiological tests together with reference strains from type culture collections and other sources. The isolates were mainly collected from the blood agar plates incubated 2 and 7 days anaerobically (2H and 7H) as described in Chapter 2. The aim of the present chapter is to present a more close identification of these isolates.

MATERIAL AND METHODS

The isolates together with 38 reference strains were examined by methods for numerical taxonomy. The reference strains and their sources are given in Table 5: 1. All the strains were maintained in the lyophilized state. During the investigation they were kept on blood agar in a refrigerator (+6°C), and subcultured each 3rd week.

The test-media and incubation time were the same as those used by Carlsson (1968 a) with the following modifications:

Growth and reduction of 0.1 per cent methylene blue milk were studied (Cowan and Steel 1965). Growth on MC-agar (Carlsson 1967 b) after incubation 48 hours in 95 per cent N₂ and 5 per cent CO₂ was recorded. Sensitivity for the antibiotics was tested with single paper discs (The Paper Disc Method 1970) supplied by AB Biodisk, Stockholm, Sweden. All the tests were run once.

During the work, it was observed that with some of the characteristics, the established groups could be separated more easily from each other than with other characteristics. The twenty-three characteristics being

Table 5: 1. *List of reference strains.*

<i>Aerococcus viridans</i>	NCTC 8251
<i>Diplococcus pneumoniae</i>	NCTC 7465
<i>Leuconostoc mesenteroides</i> **	NCDO 523
<i>Streptococcus agalactiae</i>	NCTC 8181
<i>Streptococcus bovis</i>	ATCC 9809
<i>Streptococcus cremoris</i> **	NCDO 607
<i>Streptococcus durans</i>	NCDO 596
<i>Streptococcus equinus</i>	ATCC 9812
<i>Streptococcus faecalis</i>	NCTC 8213
<i>Streptococcus facium</i> sp. no 157	The Serum Institute Copenhagen, Denmark
<i>Streptococcus lactis</i> **	NCTC 6681
<i>Streptococcus milleri</i>	NCTC 10708
<i>Streptococcus mitis</i>	ATCC 9811
<i>Streptococcus mutans</i> , sp. IB	Krasse 1966
<i>Streptococcus mutans</i> , sp. 3720	Fitzgerald and Keyes 1960
<i>Streptococcus pyogenes</i>	NCTC 8198
<i>Streptococcus salivarius</i>	NCTC 8618
<i>Streptococcus salivarius</i>	NCTC 9759
<i>Streptococcus salivarius</i>	ATCC 13419
<i>Streptococcus sanguis</i>	ATCC 10556
<i>Streptococcus sanguis</i>	ATCC 10557
<i>Streptococcus sanguis</i>	ATCC 10558
<i>Streptococcus sanguis</i> , sp. 903	Carlsson 1968 a
<i>Streptococcus</i> sp. JC 53* (Group V: A)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC 60* (Group V: A)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC 64* (Group V: A)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC 69* (Group V: A)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC72* (Group V: B)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC76* (Group V: B)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC81* (Group V: B)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC84* (Group IV)	Carlsson 1968 a
<i>Streptococcus</i> sp. JC87* (Group IV)	Carlsson 1968 a
<i>Streptococcus</i> sp. MG	ATCC 9895
<i>Streptococcus</i> sp. Group H	ATCC 8144
<i>Streptococcus thermophilus</i>	NCDO 573
<i>Streptococcus uberis</i> SS 842	Dr. Facklam, Center for Disease Control, Atlanta, Georgia 30333, U.S.A.
<i>Streptococcus uberis</i> SS 845	Dr. Facklam, Center for Disease Control, Atlanta, Georgia 30333, U.S.A.
<i>Streptococcus viridans</i>	NCTC 10712

* Refer to the number given to this strain by Carlsson (Odont. Revy 19, 137, 1968 a) in the dendrogram on page 148.

** Incubated at 30°C.

of more significance for the separation of the groups are presented in Table 5: 4. The other 45 characteristics used in the taxonomic analysis are as follows. Cell size, mean number of cells in the chains. Acid from D-galactose, D-ribose, arabinose, D-xylose, D-glucosamine HCl, glycerol, trehalose, maltose, lactose, cellobiose, salicin, arbutin, inulin (autoclaved), soluble starch, N-acetyl-glucosamine, inositol, D-mannose, fructose, rhamnose, α -methyl-D-glucoside, glycogen, dextrin. Ammonia produced from L-serine; decarboxylation of L-tyrosine; dissimilation of potassium citrate and growth at $+45^{\circ}\text{C}$. Growth in broth containing: crystal violet 0.0004 %, sodium chloride 3.0 %, methylene blue 0.1 %, thallos acetate 0.4 %, potassium tellurite 0.1 %, potassium tellurite 0.04 %. Growth at pH 5.6 in citrate-phosphate buffer. Zooglea formation in raffinose broth. Iodophilic cells in broth containing: sucrose (brown colour), sucrose (blue colour), and glucose (brown colour). Growth inhibited by: neomycin (50 $\mu\text{g}/\text{disc}$), nitrofurantoin (0.03 $\mu\text{g}/\text{disc}$), streptomycin (50 $\mu\text{g}/\text{disc}$), bacitracin (10 I.U./disc) and optochin (8 $\mu\text{g}/\text{disc}$).

In addition to the above-mentioned characteristics, all the strains were tested for Gram reaction (Preston and Morell 1962), presence of oxidase (Kovács 1956), acid from melezitose, l-fucose, alfa-methyl-mannoside, glucose, l-sorbose, adenitol, dulcitol; iodophilic cells in broth containing raffinose or glucose—blue colour reaction (Carlsson 1968 a) and sensitivity for antibiotics in paper discs containing chloramphenicol (30 $\mu\text{g}/\text{disc}$) (The Paper Disc Method 1970).

The formation of the taxonomic groups was based on 80-phenons. Thus, strains within a group or phenon displayed not less than 80 per cent similarity in the analysis used (Sneath and Sokal 1962).

Experimental errors of the tests

(In collaboration with Mr. C. J. Lamm, Department of Mathematical Statistics, University of Lund, Sweden.)

During the preparation of this manuscript, Sneath and Johnson (1972) have published a paper about the influence of errors in microbiological tests on taxonomic similarities.

The methods and reference strains used in the present study are close to those of Carlsson's investigation in 1968, and it was considered of interest to make a comparison between the results of the two studies. The results for 27 of the reference strains used in the present study (Table 5: 2) were therefore compared with Carlsson's (1968 a) results for the same strains. The results from the 25 tests presented in Carlsson's (1968 a) Table 3, were compared for each strain.

Table 5: 2. *The results in 25 tests for 27 of the reference strains in comparison with Carlsson's (1968 a) results for the same strains and tests.*

Strains	Number of tests being positive, negative and variable:					
	Comparison between Carlsson's first test results and the present results.			Comparison between the first and the second tests in Carlsson's results.		
	positive + +	variable + - or - +	negative - -	positive + +	variable + - or - +	negative - -
NCDO 523	10	2	13	9	3	13
NCDO 573	1	2	22	1	1	23
NCDO 596	11	1	13	9	1	15
NCTC 6681	13	0	12	12	0	13
NCTC 7465	6	2	17	6	2	17
ATCC 8144	6	1	18	7	0	18
NCTC 8181	6	4	15	10	0	15
NCTC 8198	9	1	15	9	1	15
NCTC 8251	2	1	22	2	1	22
NCTC 8618	6	4	15	10	1	14
ATCC 9759	7	2	16	8	2	15
ATCC 9809	14	1	10	15	0	10
ATCC 9811	4	2	19	5	1	19
ATCC 9812	9	0	16	8	1	16
ATCC 9895	11	0	14	10	0	15
ATCC 10556	7	3	15	9	1	15
ATCC 10557	2	2	21	3	3	19
ATCC 10558	9	1	15	10	0	15
J.C. 84	6	2	17	6	1	18
J.C. 64	5	2	18	5	1	19
J.C. 81	5	2	18	6	1	18
J.C. 87	4	3	18	3	3	19
J.C. 72	9	1	15	11	0	14
J.C. 76	9	1	15	9	1	15
J.C. 69	5	1	19	5	1	19
J.C. 53	1	5	19	3	3	19
J.C. 60	10	0	15	10	0	15
	187	46	442	201	29	445
	p = 3.5 per cent			p = 2.2 per cent		

The probability that a result is erroneous (an expected positive test-result is observed to be negative or an expected negative test-result is observed to be positive) can be expressed by the symbol p . If the tests are repeated, the probability of obtaining the results one positive (+) and one negative (-) or one negative (-) and one positive (+) is $2p$

$(1 - p)$. Let us regard the results in Table 5: 2 as true double tests, and equate this expression to the observed relative frequency of “+ -” and “- +”, denoted by x .

Thus we have

$$2 p (1 - p) = x$$

$$\text{which gives } p = 0.5 \pm \sqrt{\frac{1 - 2x}{4}}$$

$$\text{where } x = \frac{(+ - / - +)}{(+ +) + (- -) + (+ - / - +)}$$

When these formula are applied to a comparison between Carlsson's first test results and the present results (Table 5: 2) p was estimated to 3.5 per cent. The corresponding figure for Carlsson's double tests gave $p = 2.2$ per cent.

It may be noted that Sneath and Johnson consider 10 per cent to be the upper limit of an acceptable error probability in order to avoid an unduly large influence of erroneous results.

Our p -estimate of 3.5 per cent is a measure of the closeness between Carlsson's and our results, not an estimate of the error probability of our method.

RESULTS AND DISCUSSION

All the isolates clustered with *Streptococcus* reference strains in one of the established groups (Diagram 5: 1). Thus, the preliminary grouping of these isolates based on cell arrangement in Gram stained smears, catalase reaction, O-F-test, growth on Mitis-Salivarius agar and blood agar aerobically (Table 2: 9), were of value for the identification of the facultative anaerobic streptococci observed in this study.

Sherman (1937) divided the genus *Streptococcus* into four groups, the pyogenic, viridans, enterococcus and lactic groups. The viridans group has long been considered as heterogeneous and has been described by Drucker and Melville (1971) as “a taxonomic dumping ground for streptococcal mis-fits”. However, in recent years several studies have been concerned with the classification of the viridans streptococci (Colman and Williams 1967, 1972, Carlsson 1968 a, Colman 1968, Guggenheim 1968, Drucker and Melville 1969, 1971, Thomson 1970). Six species are now recognized, *S. pneumoniae*, *S. salivarius*, *S. mitior*, *S. milleri*, *S. sanguis* and *S. mutans*—each being serologically hetero-

Table 5:3. *Inter- and intra-group mean percentage similarity values in the numerical taxonomic analysis (80 phenon groups).*

Group Number of strains	SA 7	SB 10	SC 4	SD 12	SE 4
Group SA	85.0				
Group SB	73.4	88.1			
Group SC	69.8	76.3	81.8		
Group SD	62.2	73.5	76.8	82.5	
Group SE	65.0	75.4	70.1	76.0	83.9

geneous but possessing a distinctive combination of biochemical and physiological characteristics (Colman and Williams 1972).

To a considerable extent the classification of the heterogeneous viridans group has been clarified by means of numerical taxonomic analysis (Colman and Williams 1967, Colman 1968, Carlsson 1968 a, Drucker and Melville 1969, 1971). With the aim to identify the streptococci isolated in the present study they have also been studied by numerical analysis, using methods which have previously been employed for examination of oral streptococci (Carlsson 1968 a).

Five groups were established and designed SA—SE (Diagram 5:1). From the Diagram 5:1 and the intergroup affinities (Table 5:3), it can be observed that group SA is distinctly different from the other groups among which the similarities range from 70.1—76.8 per cent. This observation is in agreement with previous reports. Drucker and Melville (1971) found that *S. mutans* was a well defined species while *S. mitis*, *S. salivarius* and *S. sanguis* were more heterogeneous with overlappings between the three species. Colman and Williams (1967) as well as Colman (1968, 1969) observed that *S. milleri*, *S. sanguis* and *S. mitior/viridans* did not form clear-cut biological entities and Colman (1969) considered them as aggregated species from which subspecies can be constructed for special purpose.

Group SA

In group SA five isolates clustered with two reference strains of *S. mutans*. This species, first described by Clarke (1924), has been extensively studied during the last years (Fitzgerald and Keyes 1960, Krasse 1966, Carlsson 1967 a, 1968 a, Edwardsson 1968, Guggenheim 1968, de Stoppeelaar 1971, Thomson 1970, Bratthall 1970, 1972, *inter alia*). The Subcommittee on Pneumococci and Streptococci (International Committee on Nomenclature of Bacteria 1971) consider that *S. mutans* should be

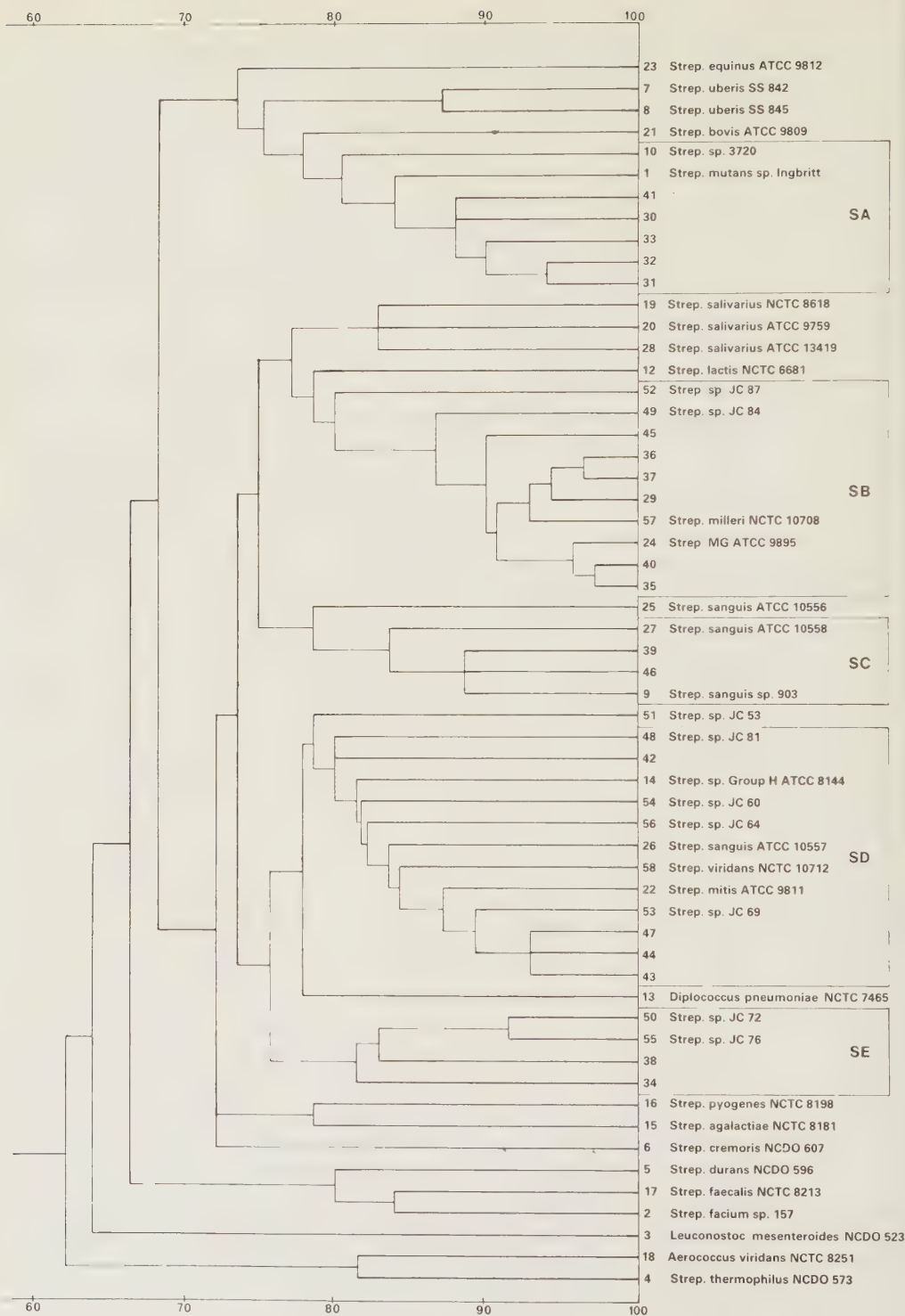


Diagram 5: 1. Dendrogram showing the resemblance among the 57 strains tested. The figure for each strain is the working designation of the strain. The identity of the reference strains are given in table 5: 1.

Table 5: 4. *Some characteristics of interest for differentiating group SA SE from each other and from the rest of the reference strains.*

Characteristics		Group					
		SA	SB	SC	SD	SE	Rest
		No. of strains					
		7	10	4	12	4	20
<i>MS-agar</i>							
Consistency of colony	soft	7	10	1	10	4	17
	firm	0	0	3	1	0	1
	mucoid	0	0	0	0	0	1
Appearance of agar surface around the colony	moat	2	0	2	1	0	1
	wrinkled	3	0	1	0	0	0
	smooth	2	10	1	10	4	17
Hemolysis	α	0	2	3	12	1	6
	β	2	0	0	0	0	3
	γ^*	5	8	1	0	3	11
pH-sucrose broth	≤ 4.1	1	1	0	0	0	0
	4.2—4.4	4	2	0	1	2	4
	4.5—4.8	2	7	4	7	2	4
Hydrogen peroxid formed:		0	4	4	12	4	5
<i>Final pH < 5.6 in broth containing:</i>							
mannitol		7	0	0	0	0	5
sorbitol		7	0	0	0	0	3
melibiose		6	0	0	4	4	4
raffinose		6	0	0	9	4	6
esculin		2	0	0	0	0	3
inulin (filtrated)		7	0	4	3	0	7
glycerol aerobic		0	0	0	0	0	1
Hydrolysis of:	esculin	7	8	3	0	0	12
	arginine	0	8	4	2	4	9
Growth in broth containing:	NaCl 4 %	7	3	1	0	0	11
	NaCl 6.5 %	1	0	0	0	0	8
	Methylene-blue 0.1 %	0	0	1	0	0	5
Growth on MC-agar		7	8	0	0	0	7
Growth at pH 5.2	acetatbuffer	6	4	0	0	2	7
	citratephosphate-buffer	5	8	1	4	4	10
Zooglea-formation in sucrose broth		7	0	4	2	0	6
Growth inhibited by:							
Bacitracin (0.2 I.U./disc)		1	1	0	5	2	9
Sulphafurazole 2.4 μ g/disc		0	2	4	12	4	11

* γ = No hemolysis.

conserved as a valid species. Some characters for differentiating this group from the others are given in Table 5: 4.

Group SB

In group SB, 6 isolates clustered with the reference strains *S. milleri* NCTC 10708 and *Streptococcus MG* ATCC 9895. With few exceptions the strains in this group fermented lactose, sucrose, trehalose, salicin, arbutin, esculin, hydrolysed arginine and esculin, grew on MC-agar and in citrate-phosphate buffer at pH 5.2 and were nonhemolytic. *S. milleri* was first described by Guthof (1956, 1960) who isolated it from oral infections. At the present time there is little information in the literature concerning the occurrence of *S. milleri* as part of the residual oral microflora. Studies currently in progress seem, however, to indicate its presence in dental plaque (Bowden *et al.* 1975, Mejåre and Edwardsson 1975).

In agreement with previous observations concerning the relationship between *S. milleri* and *Streptococcus MG* (Colman and Williams 1967, 1972) it was observed that the reference strain *Streptococcus MG* ATCC 9895 joined group B. This strain, repeatedly associated with primary atypical pneumonia, is considered as an inhabitant of normal human mouth (Swift 1952, Kraus *et al.* 1953).

Group SC

Two isolates are clustered with *S. sanguis* ATCC 10558 and *S. sanguis* sp. 903 (Carlsson 1965 a). They were found to form small zoogeic colonies of firm consistence on Mitis-Salivarius agar and produced a substance in sucrose broth which could be precipitated with ethanol. They were alpha-hemolytic, hydrolysed arginine with formation of NH_3 and produced hydrogen peroxide. Thus, they have characteristics known to be of significance for the identification of *S. sanguis* (White and Niven 1946, Carlsson 1968 a, Guggenheim 1968).

S. sanguis ATCC 10558 and *S. sanguis* sp. 903 were included by Carlsson (1968 a) in his group I: B, which he considered as *S. sanguis*. Later on Colman and Williams (1972) observed a complete agreement between Carlsson's group I: B and the cluster of *S. sanguis* from Colman's (1968) collection. Thus the information available about the isolates in group SC justify giving them the name *S. sanguis*.

Group SD

Four isolates clustered with the non-polysaccharide producing strains *S. viridans* NCTC 10712, *Streptococcus sp. Group H* ATCC 8144, and

Streptococcus *sp.* JC 60, 64, 69 and 81, but also with the dextran producing strains *S. sanguis* ATCC 10557 and *S. mitis* ATCC 9811. All twelve strains in this group were alpha-hemolytic. Ten strains appeared with soft colonies on Mitis-Salivarius agar. All produced hydrogen peroxide and were susceptible to sulphafurazole and a majority of them fermented raffinose. Few hydrolysed arginine and none esculin. Four strains were aciduric as judged by the growth at pH 5.2. Only the reference strain *S. sanguis* ATCC 10557 and *S. mitis* ATCC 9811 were zooglea forming in sucrose broth.

Dextran producing streptococci resembling *S. mitis* have been described (Niven *et al.* 1946, Guggenheim 1968). *S. sanguis* ATCC 10557 is the same strain as *S. sanguis* serotype II NCTC 7864, which is classed with *S. mitis* by Colman and Williams (1967, 1972). However, dextran producing strains resembling *S. sanguis* ATCC 10557 and *S. mitis* ATCC 9811 form a homogeneous group as observed in studies of their overall similarities and nutritional requirements (Carlsson 1968 a, 1972). It may therefore be appropriate to separate them from the non-poly-saccharide producing strains of the mitis group. The clustering of these dextran producing strains within the present group may be due in part to the fact that only two such strains were studied.

Schottmüller (1903) proposed the name *S. mitior* for *Streptococcus* strains producing "greening" on blood agar. This term has now been reintroduced by Colman and Williams (1972) for certain strains previously labelled *S. mitis* and *S. viridans*, such as *S. viridans* NCTC 10712. The reference strains *S. sp.* JC 60, 64 and 65 belong to group V: A in Carlsson's (1968 a) collection. These were considered to bear a close resemblance to *S. mitis* as described by Sherman *et al.* (1943). Colman and Williams (1972) compared Carlsson's (1968 a) *S. mitis*-like group V: A with Colman's (1968) *S. mitior*-like strains and found them to be closely similar. Thus the information available about the isolates in group SD would appear to justify giving them the name *S. mitior*.

Group SE

Two isolates joined *S. sp.* JC 72 and *S. sp.* JC 76 and formed group SE. No named reference strains were included. The strains were found to produce hydrogen peroxide and to ferment melibiose and raffinose. They hydrolysed arginine, grew at pH 5.2, and were susceptible to sulphafurazole. No zooglea formation in sucrose broth was observed and none of the strains hydrolysed esculin. The colonies on Mitis-Salivarius agar were soft with no change in the surface of the agar surrounding the colonies. Carlsson (1968 a) considered his group V: B, including *S. sp.*

JC 72 and 76, as resembling *S. mitis* as described by Sherman *et al.* (1943). Colman and Williams (1972), on the other hand, considered a cluster with characteristics as described for Carlsson's group V:B as a variety of *S. sanguis* irrespectively of their inability to form polysaccharide from sucrose.

In the present study, group SE showed higher intergroup similarity with *S. mitior* than with *S. sanguis* and based on the observations that polysaccharide producing strains of *S. mitior/mitis* form a homogeneous group, as mentioned above, the isolates in group SE were considered as resembling those of *S. mitior*.

The reference strain *S. pneumoniae* NCTC 7465 showed a similarity of 78 per cent with group SE. The observation of this relationship is supported by previous findings made by Austrian *et al.* (1972), who reported a close relationship between the well recognized species of *S. pneumoniae* (Colman 1968) and "viridans"-streptococci based on serological studies of properties of the cell wall polysaccharides as well as deoxyribonucleic acid-(DNA-)transformation among strains of the two species.

None of the isolates clustered around the reference strains of *S. salivarius*. Nor was any isolate observed which formed large mucoid colonies on Mitis-Salivarius agar, which has been considered as a significant character for strains resembling the species *S. salivarius* (Sherman *et al.* 1943, Chapman 1944, Williams 1956).

SUMMARY

The properties of nineteen Gram positive, catalase negative cocci which formed chains when growing in liquid media are described. These isolates, together with 38 reference strains of the genus *Streptococcus*, were subjected to numerical taxonomic analysis. Of the 85 biochemical and morphological tests employed initially, 68 were selected for inclusion in the numerical analysis. All the isolates were clustered into one of five groups within the genus *Streptococcus*. These clusters resembled *Streptococcus mutans* (5 isolates), *Streptococcus milleri* (6 isolates), *Streptococcus sanguis* (2 isolates) and *Streptococcus mitior* (*mitis*) (2 clusters, 6 isolates).

None of the isolates resembled the species *Streptococcus salivarius*, *Streptococcus pneumoniae*, *Streptococcus pyogenes* or *Streptococcus agalactiae*.

6. Identification of Gram negative anaerobic cocci and rods

Ten isolates were Gram negative. At the primary culturing these isolates grew only in TA-Ser medium (Möller 1966) and on blood agar incubated in 94 per cent H₂ and 6 per cent CO₂, some of them with only meagre growth. Six coccus-like isolates were separated from four rod-like ones. The coccus-like isolates resisted decolorization when Gram stained smears were prepared, and some of them were therefore initially mistaken for Gram positive cocci. This group also included one anaerobic isolate which at the preliminary identification (Chapter 2, pp. 37—42) was assigned to identification group I.

This chapter concerns closer identification of the isolates.

MATERIAL AND METHODS

The isolates were stored in the freeze-dried state until used.

The Gram reaction was tested as described in Chapter 2 and with Kopeloff modification, as described by Holdeman and Moore (1972). To check the reliability of the method, young cultures of a Gram positive strain, *Staphylococcus aureus* NCTC 8532, and a Gram negative organism, *Neissera sp.* (Gram negative, aerobic diplococci being catalase and oxidase positive; Cowan and Steel 1965) were stained on the same slide as the isolates (Bartholomew and Mittwer 1952). Smears were prepared from both young and old cultures in TA-Ser medium and from pre-reduced anaerobic, sterilized (PRAS) media containing peptone, yeast extract and glucose (PYG according to Holdeman and Moore 1972). The size of the cells was measured in Gram stained smears with an eyepiece micrometer (Leitz).

The cell shape and motility were studied in wet preparations (Holdeman and Moore 1972) with darkfield and phase contrast microscope. The smears were taken from the same cultures as described above for the Gram stain preparations. For the photographic reproduction material was mixed with a water solution containing 0.3 per cent (w/v) of Bacto agar.

The rod-like isolates were also examined in electron microscope (Philips EM 300) by Dr. B. Sundström, Dept. of Oral Histopathology, School of Dentistry, Malmö. Eight to twenty-four hour cultures in TA-Ser medium (Möller 1966), PY-base, PY-glucose or chopped meat (Holdeman and Moore 1972) were fixed with 1 per cent (w/v) osmium tetroxide in 0.02 M sodium-phosphate buffer solution pH 7.2; centrifuged and washed twice in distilled water. Samples were then examined unshadowed.

The capacity to grow and produce gas was tested in Veillonella agar (Difco 0917—01) prepared in the way described for the oxidation-fermentation test (O-F-test) by Evans *et al.* (1965). A large inoculum of a culture in TA-Ser medium was used. Growth and gas production were recorded after 4 and 7 days.

Fermentation products from glucose, lactate and pyruvate were analysed by Professor J. Carlsson, Dept. of Oral Microbiology, University of Umeå, as described in Chapter 4. Incubation time before analysis was 14 days. The chromatographic analyses were performed from PY-broth with glucose, lactate and pyruvate (Holdeman and Moore 1972) as modified by Carlsson (1973).

The further characterization was based on certain biochemical reactions in PRAS-media (Holdeman and Moore 1972). These tests are summarized in Tables 6: 1 and 6: 2.

RESULTS AND DISCUSSION

Identification of coccus-like isolates

Six coccus-like isolates were Gram negative and 0.7—0.9 μm in diameter. The cells appeared in clusters or irregular masses. All of them grew and produced gas in Veillonella agar.

Lactate and pyruvate were converted to propionic and acetic acids (Table 6: 1). They did not ferment glucose or any of the other carbohydrates tested. All of them were nitrate positive and produced gas in PY-broth with glucose and agar (PYG agar deep according to Holdeman and Moore 1972). Four of the isolates decomposed H_2O_2 .

The Gram negative anaerobic cocci have recently been transferred from *Neisseriaceae* to *Veillonellaceae* fam. nov. (Rogosa 1971 d). The new family includes three genera, *Veillonella*, *Acidaminococcus* and *Megasphaera*. The six isolates resembled species of genus *Veillonella*. In contrast to *Megasphaera spp.* (Rogosa 1964, 1971 e), the isolates did not ferment any carbohydrates and did not produce butyric, valeric or caproic acid. The fermentation of lactate separates them from *Acidaminococcus spp.* (Rogosa 1964, 1965, 1971 d). Four of them were

Table 6: 1. *Biochemical characteristics of anaerobic Gram negative cocci.*

Characteristics	Veillonella		Number of isolates with characteristics	
	parvula ATCC 17744	alcalescens ATCC 17746	4	2
Acid end products from				
glucose	—	—	—	—
lactate	A, P ¹	A, P ¹	A, P ¹	A, P ¹
pyruvate	A, P ²	A, P ²	A, P ²	A, P ²
Carbohydrate				
fermentation ³	—	—	—	—
Nitrate reduction	+	+	+	+
Decomposition of H ₂ O ₂	—	+	+	—
Gas in PYG-agar deep	+	+	+	+

Symbols

+ = positive reaction; — = negative reaction; A = acetic acid; P = propionic acid.

¹ The proportion of acetic to propionic acid was approx. 1 : 1.

² The proportion of acetic to propionic was approx. 5 : 1.

³ None of the reference strains or isolates fermented arabinose, cellobiose, erythritol, esculin, fructose, glucose, lactose, maltose, mannitol, mannose, raffinose, salicin, sorbitol, starch, sucrose, trehalose or xylose.

regarded as *Veillonella alcalescens* because of their ability to decompose H₂O₂ and the other two isolates, which did not decompose H₂O₂, were identified as *Veillonella parvula* (Rogosa 1964).

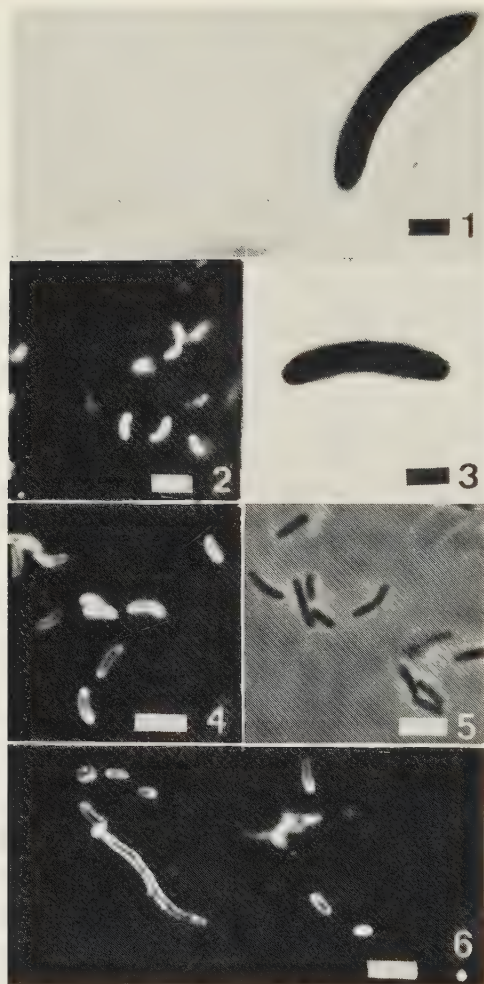
Identification of rod-like isolates

Four isolates were found to be Gram negative rods. None of the isolates grew in *Veillonella* agar.

A. CURVED RODS

Three isolates (111C1, L111C2 and L111C5) appeared in Gram stained smears with slightly curved (kidney-like) cell shape and sometimes slightly S-shaped forms. The ends of the cells were rounded. They were 5—6 μ m long and 1 μ m in diameter; larger forms up to 10—12 μ m were also observed. In wet preparations mainly the same curved forms were observed (Figs 6: 2—4).

Motility of one isolate (L111C2) was a constant observation in the wet preparations from young cultures and one terminal flagella was also detected with the electron microscope (Fig. 6:1). There was no clear evidence of motility of the other two curve-shaped isolates. The



Figs. 6: 1—6. The cell morphology of Gram negative rods.

(1): Electron photomicrograph of isolate L111C2 from PY-broth culture after 2 days incubation. Black bar indicate 2 μ m. Photographed at $\times 8000$. (Courtesy Dr. Sundström.)

(2): Darkfield preparation of isolate L111C2 from TA-Ser medium after 2 days incubation. White bar indicate 5 μ m. Photographed at $\times 1000$.

(3): Electron photomicrograph of isolate L111C1 from PY-broth culture after 2 days incubation. Black bar indicate 2 μ m. Photographed at $\times 8000$. (Courtesy Dr. Sundström.)

(4): Darkfield preparation of isolate L111C1 from TA-Ser medium culture after 2 days incubation. White bar indicate 5 μ m. Photographed at $\times 1000$.

(5): Wet preparation of isolate L116D5 from TA-Ser medium culture after 2 days incubation. Photographed in phase contrast microscope at magnification $\times 1000$. White bar indicate 5 μ m.

(6): Darkfield preparation of isolate L116D5 from blood agar culture after incubation in 94 per cent H_2 and 6 per cent CO_2 for 4 days. White bar indicate 5 μ m. Photographed at $\times 1000$.

motility of the isolate 111C1 was not constantly observed but when it was, few cells showed a clear swimming movement. As for the third isolate, colonies observed at the primary isolation showed a surface translocation which might have been a flagella-dependent swarming or swimming (Henrichsen 1972). On the other hand, no motility could be detected in the wet preparations. Both isolates 111C1 and L111C5 showed no flagella in studies with electron microscope or in wet preparations stained for flagella (Leifson 1961).

In so far as these three isolates had a Gram negative curved cell shape, produced acetic and propionic acids and failed to produce butyric acid they fitted in with the description given by Macdonald *et al.* (1959) and Loesche and Gibbons (1965) for oral members of *Spirillum* or *Selenomonas*. The pattern of acid end products distinguished them from *Vibrio* (Loesche and Gibbons 1965, Holdeman and Moore 1972). At least two of the isolates also resembled *Spirillum/Selenomonas* in motility. However, the lack of flagella or the presence of polar or peritrichous flagella is fundamental in classification and identification of bacteria (Breed *et al.* 1957, Kingsley and Hoeniger 1973). Members of genus *Selenomonas* should have flagella near the center of the concave side (Breed *et al.* 1957, Kingsley and Hoeniger 1973), while those of genus *Spirillum* are bipolarly flagellated and are regarded as microaerophilic and occur mainly in marine environments (Hylemon *et al.* 1973). Thus, despite the resemblance between *Spirillum sputigenum* (Macdonald *et al.* 1959, Loesche and Gibbons 1965) and the present isolates, it is not quite obvious from the literature whether they could be assigned to this genus. Furthermore, as two of the isolates showed no motility with certainty, all three were only tentatively regarded as anaerobic curve-shaped rods within *Spirillaceae* (Breed *et al.* 1957).

B. REGULAR STRAIGHT ROD

In wet preparations and Gram stained smears the isolate L116D5 was found to form straight rods, sometimes filamentous of varying length (mainly 2.0—7.0 μm) and “not predominating curved” (Smith and Holdeman 1968). The impression was that short cells were present mainly in the microscopic preparations. The isolate produced succinic acid, lactic acid and acetic acid, and may therefore be regarded as member of *Bacteroides* (Loesche and Gibbons 1965, Holdeman and Moore 1972). The isolate did not produce black pigment, it did not grow in bile medium or ferment arabinose, but it converted mannose to acid. Further characteristics are given in Table 6:2. Based on these observations and the demonstration of lactic acid production this isolate

Table 6: 2. *Biochemical characteristics of Gram negative anaerobic rods.*

Characteristics	Isolates			
	L111C2	111C1	L111C5	L116D5
Motility	+	(+) ²	(+) ²	—
Acid end products from				
glucose	A, P	A, P, L	A, P, L	A, L, S
lactate	A, P ¹	—	A, P	—
pyruvate	A, P	A, P	A, P, L	A, IV
Terminal pH in glucose broth	5.5	5.4	4.5	4.8
Nitrate reduction	+	+	+	—
Growth in bile medium	—	—	NT	—
Starch hydrolysis	—	—	—	+
Gelatine liquefaction	—	—	—	+
Esculin hydrolysis	—	—	—	+
Acid from				
cellobiose	—	—	—	+
inositol	—	—	+	—
mannitol	—	+	+	—
melezitose	—	—	+	—
melibiose	—	—	+	+
rhamnose	—	—	+	—
ribose	—	—	+	—
sorbitol	—	+	+	—
starch	—	—	+	+
trehalose	—	—	+	—

Symbols

+ = positive reaction and — = negative reaction. A = acetic acid, IV = isovaleric acid, L = lactic acid, P = propionic acid, S = succinic acid, NT = not tested.

¹ All lactic acid disappeared.

² See text.

All isolates fermented fructose, lactose, maltose, mannose, raffinose, sucrose, but not arabinose erythritol, esculin, salicin and xylose. All produced gas, but none H₂S or indol.

was regarded as *Bacteroides oralis* (Loesche *et al.* 1964, Holdeman and Moore 1972).

SUMMARY

Six cocci and four rods, all being Gram negative anaerobic isolates, were described and identified. Four of the isolates resembled *Veillonella alcalescens* and two *Veillonella parvula*. Three of the isolates were identified as Gram negative curved rods within *Spirillaceae*. The characteristics of one rod-like isolate agreed with those of genus *Bacteroides oralis*.

7. Identification of certain Gram positive cocci, coccobacilli and rods

Sixteen Gram positive isolates could not be referred to any of the groups described in Chapters 3—6. This group of organisms included five isolates assigned to group I at the preliminary examination (Chapter 2).

This chapter concerns a closer identification of these isolates.

MATERIAL AND METHODS

The isolates were stored in the freeze-dried state until used.

A. ANAEROBIC ISOLATES

This group consisted of cocci (8 isolates) and coccobacilli (3 isolates).

The Gram reaction, cell shape, analysis of fermentation products from glucose, lactate and pyruvate and the biochemical PRAS-tests were determined in the way described in Chapter 6. The three rod-like isolates were further tested for stimulation of growth in PYG supplemented with bile, Vitamin-K-Hemin-solution and serum (Holdeman and Moore 1972).

B. FACULTATIVE ANAEROBIC ISOLATES

This group consisted of cocci (4 isolates) and rods (1 isolate).

All the tests for these isolates were performed under aerobic conditions with biochemical tests, as described in Chapter 3. In all the broths media, cysteine HCl was omitted. Oxidation/fermentation of glucose was studied with both the media and the methods described by Evans *et al.* (1965) and by Hugh and Leifson (1953). Anaerobic utilization of mannitol was studied as described by Evans *et al.* (1965). Studies on coagulase and phosphatase production were performed with the media and methods described in the manuals of Difco Laboratories (1968) and Oxoid Ltd. (1965), respectively.

The single facultative anaerobic rod was further tested for spore-formation as described in Chapter 3.

RESULTS AND DISCUSSION

Identification of anaerobic cocci

A. ISOLATES WITH CELLS ARRANGED IN PAIRS AND CHAINS

Six isolates were Gram positive cocci in smears from PY-glucose cultures. They were round to slightly elongated. The average size was between 0.6–0.9 μm , but ovoid forms up to 1.6 μm could be observed.

At the primary examination four of these isolates had been provisionally identified as short Gram positive pleomorphic rods and were therefore assigned to group I. But the further studies with the prereduced media, described in the present chapter, revealed that these isolates were more similar to cocci. It is known that certain environmental changes can induce morphological abnormalities in bacterial cells (Rogers 1970, Rosan and Eisenberg 1973). A higher oxidation-reduction potential in the media used at the primary identification than that existing in PRAS-media (Holdeman and Moore 1972) might thus have been one possible explanation for the observations.

Three isolates (L104C4, L111C4, L116D2) often appeared in pairs and sometimes in short chains. They were stimulated by Tween 80. Analysis of the acid end product of cultures in PY-glucose revealed that the ratios between lactic acid, acetic acid and ethanol were 15 : 1 : 1. They utilized pyruvate with the production of acetic and lactic acids (2 : 1). L104C4 also produced small amounts of succinic acid. The pattern of carbohydrate fermentation and reactions in other biochemical tests are given in Table 7:1.

The other three isolates (43CT, 55CT, L108B1) formed cocci, sometimes slightly elongated and arranged mainly in chains of varying length in cultures in PY-glucose. Coccoid rod-like forms were also found. They produced lactic acid, acetic acid and ethanol in PY-glucose in the ratios 10 : 1 : 1. Pyruvic acid was utilized with the production of acetic acid and lactic acid (4 : 1), and acetic acid and ethanol (1 : 1) were produced from lactic acid. Tween 80 stimulated the growth of isolate L108B1.

These six Gram positive anaerobic cocci had properties suggesting that they might be members of *Peptococcaceae* or *Streptococcus*. The production of lactic acid separated them from *Peptococcaceae* and assigned them to genus *Streptococcus* (Rogosa 1971 b, Holdeman and Moore 1974). The characteristics of the three isolates L104C4, L111C4 and L116D2 (Table 7:1) were closely related to each other and resembled those of *Streptococcus constellatus* as described by Holdeman and Moore (1972, 1974). They were therefore identified as *Streptococcus constellatus*.

Table 7: 1. *Biochemical characteristics of anaerobic streptococci.*

Characteristics	Streptococcus			Streptococcus		
	Isolates:			Isolates:		
	L104C4	L111C4	L116D2	constellatus Holdeman and Moore (1974)	7 strains	intermedius Holdeman and Moore (1974)
	L104C4	L111C4	L116D2	43CT	55CT	L108B1
Acid from						63 strains
cellobiose	a	a	w	a	a	aw
esculin	-	-	-	w-	w	-a
lactose	-	-	-	wa	a	a
mannitol	-	-	-	a	aw	-a
melezitose	-	-	w	-	-	-w
melibiose	-	-	-w	w	aw	w
raffinose	-	-	-	a	a	w
ribose	-	-	-	w	w	-a
salicin	a	aw	-w	a	a	a-
starch	-	-	-	-	-a	w
Esculin hydrolysis	+	+	+	+	+	+
Gelatine digestion	-	-	-	+	w+	-w
Milk	-	-	-	-	co	cu
Gas production	-	-	-	+	+	-
Nitrate reduction	+	+	+	-	-	ND

Symbols

+ = positive reaction. - = negative reaction. a = acid production in carbohydrate culture, pH < 5.5. w = weak reaction or pH between 5.5 and 6.0. co = clot. cu = clear whey with solid clot. Where two reactions are given (e.g. "+ -") the first was the more usual and the second was observed less frequently. ND = no data available.

All isolates fermented fructose, glucose, maltose, mannose and sucrose. None fermented adonitol, amygdalin, arabinose, dulcitol, glucogen, inositol, rhamnose, sorbitol and xylose. None produced indol or hydrolysed starch.

The characteristics of the isolates 43CT, 55CT and L108B1 were also almost identical (Table 7:1). They were separated from the isolates resembling *Strep. constellatus* by their regular occurrence in chains, fermentation of lactose and melibiose and their inability to reduce nitrate. The isolates 43CT, 55CT and L108B1 resembled *Streptococcus intermedius* (Holdeman and Moore 1974) and were identified as members of this species.

B. ISOLATES WITH CELLS IN PAIRS AND TETRADES

One isolate (115B1) formed large sometimes ovoid cells (1.0—2.0 μm) in Gram stained smears from PY-glucose cultures. These cells often occurred in pairs and tetrads. Arrangement of the cells in three-dimensional cubical packets was not observed at any appreciable level. No motility was detected. Growth in PRAS media was stimulated by Tween 80 but in the carbohydrate tests no drop in pH below 6.0 was observed. In PY-base cultures the amino acids were utilized with the production of acetic, propionic and butyric acids in the ratios 1 : 1 : 3. The ratios between acetic, propionic and butyric acid in PY-glucose cultures were 1 : 2 : 8 and after 14 days incubation the pH fell only to 6.3. Nitrate was not reduced; no catalase, H_2S , gas or indol was produced and no growth was detected in PY-glucose broth with Tween 80 at pH 2.5. The appearance of the cells, production of butyric acid and growth stimulation by Tween 80 corresponded to descriptions given for the Gram positive organisms "*Gaffkya anaerobia*" (Holdeman and Moore 1972). The isolate 115B1 differed from *Sarcina* in the appearance of its cell and inability to grow at pH 2.5 (Canale-Parola 1970, Rogosa 1971 c). In contrast with the description of both *Sarcina* and the Gaffkya-group, the isolate 115B1 failed to decrease the pH below 6.3 in any of the carbohydrates in the PRAS-tests.

One isolate (68CTB) formed small and sometimes ovoid cocci (0.6—1.0 μm) many of which occurred in pairs in Gram stained smears and wet preparations. Scanty growth was observed in the PRAS-media.

A slight stimulation of growth was demonstrated when the media were supplemented with Tween 80. The best growth was obtained on blood agar incubated 4 days anaerobically. No production of acids was observed in PY-glucose, PY-lactate or PY-pyruvate cultures. The pH never fell below 6.3. Nitrate and gelatine were not split; catalase, H_2S , gas or indol were not produced.

Anaerobic cocci with characteristics similar to those of isolates 115B1 and 68CTB have been described by Thomas and Hare (1954) and Hare (1967). Further studies of such properties as the capacity to utilize

peptones, amino acids, organic acids etc. (Hare 1967, Rogosa 1971 b) may be necessary for closer identification. Both isolates were tentatively regarded as anaerobic micrococci within *Micrococcaceae* (Breed *et al.* 1957).

Anaerobic rods

The cells of three isolates 63DT, L63D2 and L68C2 could be described as small cocco-bacilli or coccoid rods (about 0.5 μm to 1.0–2.0 μm). The Gram reaction was variable both in cultures from TA-Ser medium, PY-glucose broth and blood agar and was not related to the age of the cultures. Gram's stain was retained better with the Kopeloff modification (Holdeman and Moore 1972). No motility was observed and the cultures were catalase negative.

They grew very poorly in the PRAS-media and the pH never fell below 6.3 in the fermentation tests. No acid end products were observed in PY-base or PY-glucose cultures. Neither lactate nor pyruvate was utilized, threonine was not converted to propionate, no gas was formed. The growth was not stimulated by PYG-bile, PYG-Tween 80, PYG-Vitamin-K-Hemin-solution or PYG-serum and no growth was observed in PYG-bile. No nitrate was reduced and no gelatine was digested. Thus, few characteristics of value for the identification were observed. The isolates 63DT, L63D2 and L68C2 are therefore described only as Gram positive anaerobic rods.

Facultative anaerobic cocci

Four isolates were Gram positive cocci with almost spherical cells approximately 1.0 μm in diameter and appearing mainly in clusters or pairs. All produced catalase.

One (96B3) of these isolates formed elongated cells, 0.8 to 2.0 μm in diameter in media with low oxidation-reduction potential, such as TA-Ser medium and PY-glucose. Glucose was oxidized both in the standard medium recommended by the Subcommittee of Taxonomy of Staphylococci and Micrococci (1965) and in the medium suggested by Hugh and Leifson (1953). The isolate 96B3 was therefore assigned to *Micrococcaceae*, genus *Micrococcus* (Breed *et al.* 1957, Cowan and Steel 1965, Evans *et al.* 1965). The further identification followed the classification of micrococci presented by Baird-Parker (1963, 1971). The essential characteristics observed (Table 7:2) were the production of acetoin, acid production from maltose, but not from lactose, mannitol and xylose. It is therefore possible to regard isolate 96B3 as belonging to

Table 7: 2. *Some biochemical characteristics of facultative anaerobic Gram positive cocci and rods.*

Characteristics	Isolates				
	Cocci				Rod
	L58B1	L64E3	96B2	96B3	L62D3
Spore formation	NT ¹	NT	NT	NT	+
Oxidation/Fermentation of glucose	F ²	F	F	O ²	F
Coagulase production	- ³	-	-	-	NT
Acid production from					
arabinose	-	-	-	-	-
cellobiose	-	-	-	-	+
galactose	+	-	+	-	+
glycerol	-	-	-	-	-
inositol	-	-	-	-	-
lactose	+	-	+	-	-
maltose	+	+	+	+	+
mannitol	-	-	-	-	-
raffinose	-	-	-	-	-
salicin	-	-	-	-	+
xylose	-	-	-	-	-
Phosphatase production	+	-	+	-	NT
Voges-Proskauer's (VP)-test	+	+	+	+	+
Esculin hydrolysis	-	-	-	+	+
Gelatine split	+	-	-	+	-
Egg-yolk agar (clearing)	-	-	-	-	-
Nitrate reduction	+	-	-	+	+
H ₂ S-production	-	-	-	-	-

¹ NT = not tested.

² F = fermentation; O = oxidation.

³ + = positive reaction, - = negative reaction.

None of the isolates utilized mannitol anaerobically or produced coagulase.

Micrococcus subgroup 1 (Baird-Parker 1963) or *Micrococcus saprophyticus* (Baird-Parker 1970, 1971).

Three coccus-like isolates (L58B2, L64E3, 96B2) fermented glucose anaerobically in the O-F-test. All three fermented maltose, but none of them utilized mannitol anaerobically or produced coagulase. Isolate L58B2 and 96B2 produced phosphatase and fermented lactose and were therefore considered as *Staphylococcus epidermidis* biotype II, while L64E3 which was inactive in these tests was considered as *Staphylococcus*

epidermidis biotype III (Baird-Parker 1963, 1971, Cowan and Steel 1965).

Facultative anaerobic rods

One isolate (L62D3) was a Gram positive, catalase positive, motile rod, which produced subterminal spores. Glucose was fermented. Further characteristics are given in Table 7: 2. The isolate L62D3 was considered as *Bacillus* *sp.* (Cowan and Steel 1965).

SUMMARY

Sixteen isolates which could not be assigned to any of the previously described groups (Chapters 3—6) are characterized in further details and identified.

Six Gram positive anaerobic isolates could be divided into two groups with three isolates in each. The members of the groups resembled the recently described anaerobic species within *Streptococcus* viz. *S. constellatus* and *S. intermedius*, respectively.

Two anaerobic cocci were regarded as anaerobic micrococci.

Three isolates were described only as Gram positive anaerobic rods.

Of the five facultative anaerobic isolates, three were identified as *Staphylococcus epidermidis*, one as *Micrococcus saprophyticus* and one as *Bacillus* *sp.*

8. Main results and discussion

In the previous chapters a description is given of how the isolates were divided into five groups and how the members of each group were further characterized. The observations may be summarized as follows:

Group I consisted of isolates, which were assigned to one or the other of the genera *Actinomyces*, *Arachnia*, *Bifidobacterium*, *Eubacterium*, *Propionibacterium* and *Rothia*.

Group II—isolates were identified as *Lactobacillus spp.*

Group III contained isolates identified as *Streptococcus spp.*

Group IV consisted of isolates resembling those of the genera *Veillonella*, *Bacteroides* and family *Spirillaceae*.

Group V. This group was composed of a number of isolates resembling anaerobic streptococci, anaerobic micrococci, Gram positive anaerobic rods, *Staphylococcus spp.*, *Micrococcus spp.* and *Bacillus spp.*

The aim of this chapter was to describe and discuss the identified isolates in relation to their incidence within the teeth examined.

Identification and incidence of bacteria

The sampling technique was based on the removal of successive samples of dentine material from the pulp chamber towards the lesion. When reaching the soft necrotic area which was considered to represent the fully developed lesion—no further material was taken out. The first positive culture was regarded as representing the bacteria of the advancing front of carious dentine. This culture was studied further. In previous studies of the bacterial flora of deep carious dentine, this technique of approaching the dentine lesion has been used only to a limited extent (see Chapter 1, pp. 13—14).

During the preparation of the present work, however, studies were undertaken with a somewhat similar method in an endeavour to reach the bacterial front line in carious dentine (McKay 1969, Sumney and Jordan 1974). The findings in the present study that the predominant organisms were Gram positive bacteria are supported by the observations

of McKay, Sumney and Jordan as well as by several previously mentioned studies (see Chapter 1, pp. 7—12), where other sampling techniques have been used. Thus, independently of the sampling technique, available evidence seems to indicate that the environment created in deep carious dentine may favour the growth of certain Gram positive bacteria. This is in contrast with the flora in dental plaque (Gibbons *et al.* 1964, Bowden *et al.* 1975) and in infected root canals (Möller 1966, Kantz and Henry 1974), which also include a large proportion of Gram negative bacteria.

It might be questioned whether the culturing technique applied in the present study could be the reason for the low incidence of Gram negative bacteria observed. However, similar culturing techniques have been used in other studies at the department. Mejäre (1974, personal communication) in an investigation on the bacterial flora of infected root canals and Mannerberg and Edwardsson (unpublished data) in work on the bacterial flora in plaque on silicate cements regularly observed Gram negative bacteria such as *Veillonella spp.*, *Bacteroides spp.* and *Fusobacterium spp.* On the other hand, certain strict anaerobes, in accordance with the definition given by Loesche (1969), such as *Treponema spp.* would not have been able to grow out on the media. Thus, the low incidence of Gram negative bacteria can presumably only to a small extent be explained by the culturing technique. Differences in other respects between Gram positive and Gram negative bacteria, such as aciduric properties, resistance to mechanical disruption and tendency to undergo autolysis (Lamanna *et al.* 1973) may help to explain the selection of the flora in deep carious dentine.

The distribution of bacteria of different species in relation to the culturing procedures is given in Table 8: 1. Most of the data available about each tooth can be studied by comparing the data given in this table and in Table 2: 2 (see Chapter 2, pp. 40—41).

The number of positive isolation of bacteria is given in Figure 8: 1. Gram positive rods were most common (43 out of 46 teeth) followed by Gram positive cocci (15 teeth). Gram negative rods were demonstrated in only two of the teeth studied.

An attempt was made to analyse the quantitative representation of the different genera and species. This analysis was based on the establishment of the relative proportion of "representative colonies" (Socransky 1970), as observed on the blood agar plates used in the culture of dentine material. The limitations and problems connected with this technique of sampling material have previously been discussed (Socransky 1970, Chapter 2, pp. 45—46). The results of the quantitative analyses for each tooth are given in detail in Table 8: 1

Table 8: 1. *Distribution of the identified isolates on the teeth in relation to the culturing procedures.*

2A	Blood agar incubated 2—3 days aerobically.										
2H	Blood agar incubated 2—3 days in H ₂ + 5 % CO ₂ .										
7H	Blood agar incubated 7 days in H ₂ + 5 % CO ₂ .										
T	TA-Ser medium incubated for up to 14 days.										
CFU	Total number of colony forming units (CFU) on each blood agar plate.										
%	The relative proportion for the colony forming units (CFU) of each identified morphological form in relation to the total number of CFU on each plate.										
	Tooth no. and portion	2A	2H			7H			T		
		Genus/species	%	CFU	Genus/species	%	CFU	Genus/species	%	CFU	Genus/species
37 C											Bifidobact. sp.
38 B								Propionibacteriaceae	100	1	
40 B								Bifidobact. sp.	70	7	Strep. mutans
								Propionibact. acnes	15		
								Strep. mutans	15		
42 C											Gram positive anaerobic rod*
43 C											Strep. intermedius
44 D	Lbc sp.	100	23	Lbc casei var. rhamnosus	100	34	Lbc casei var. rhamnosus	100	34	Lbc sp.	
46 B								Gram positive rod*	100	1	Arachnia sp.
47 C				Eubact. alactolyticum	100	14	Propionibact. acnes	91	89		
							Arachnia sp.	9			

48 B								Eubact. alactolyticum
49 B								Lbc sp.
50 B								Arachnia sp.
51 C								Arachnia sp.
55 C								Strep. intermedius
58 B								Lbc fermentum
62 D								Lbc sp.
63 D								Gram positive anaerobic rod
64 E								Arachnia sp.
68 C								Strep. mitior
72 E								Anaerobic micrococci

* Lost.

Tooth no. and portion	2A		2H		7H		T	
	Genus/species	%	CFU	Genus/species	%	CFU	Genus/species	Genus/species
77 D								Lbc cellobiosus
78 C				Lbc casei var. rhamnosus	100	23	Lbc casei var. rhamnosus	40 Lbc casei var. rhamnosus
79 B							Eubact. alactolyticum	2 100
80 C							Arachnia sp. Propionibact. acnes	75 25 4
81 C	Lbc casei var. rhamnosus	100	1					Lbc casei var. rhamnosus
82 D								Strep. mutans
85 D	Lbc sp.	100	163	Lbc brevis Bifidobact. eriksonii Lbc acidophilus	60 34 6	568	Lbc brevis Bifidobact. eriksonii Gram positive anaerobic cocci* Eubact. alactolyticum	79 1300 13 7 1
86 C	Lbc sp.	100	88	Lbc casei var. rhamnosus Lbc acidophilus	82 18	630	Lbc casei var. rhamnosus Lbc acidophilus Veillonella alcalescens	536 67 32 1

88 B		Eubact.	100	1	Eubact.	66	3	Eubact.
		alactolyticum			alactolyticum			alactolyticum
					Propionibact. acnes	34		
96 B		50 3	50	2	Bifidobact. sp.	88	16	Strep. mutans
	Strep. mutans				Lbc fermentum	6		Lbc fermentum
	Strep. mitis**	25	50		Gram negative			
	Micrococcus				anaerobic cocci*	6		
	saprophyticus**	25						
97 B	Lbc cellobiosus	100	100	3	Lbc acidophilus	100	1	
102 B	Lbc sp.	100	100	29	Lbc casei		57	Lbc casei
		30			var. rhamnosus	48		
					Lbc casei	52		
103 C	Lbc casei	109	59	198	Lbc plantarum	55	105	Lbc casei
	var. rhamnosus	57			Lbc buchneri	31		var. rhamnosus
	Lbc acidophilus	43	22		Lbc casei			
			18		var. rhamnosus	11		
			1		Lbc casei	3		
104 C			66	3	Arachnia sp.	63	253	Strep. milleri
	Strep. milleri				Actinomyces israelii	24		
	Actinomyces		34		Strep. milleri	5		
	naeslundii				Strep. constellatus	4		
					Actinomyces sp.	2		
					Bifidobact. sp.	2		
106 B	Lbc sp.	100	59	198				
		202	64	514	Lbc acidophilus	97	734	Lbc sp.
			34		Lbc brevis			
					Lbc casei			
			2		var. rhamnosus	3		
					var. rhamnosus			

* Lost.

** Grew together in the same CFU.

Tooth no. and portion	2A			2H			7H			T		
	Genus/species	%	CFU	Genus/species	%	CFU	Genus/species	%	CFU	Genus/species	%	CFU
108 B				Lbc acidophilus	100	4	Strep. intermedius Lbc acidophilus	75 25	8	Lbc acidophilus		
109 D	Lbc sp.	100	24	Lbc casei Bifidobact. eriksonii	90 10	31	Eubact. alactolyticum Lbc casei Bifidobact. eriksonii	61 29 10	119	Lbc casei		
110 B	Strep. sanguis	100	1	Strep. mitior Actinomyces odontolyticus	50 50	2	Actinomyces israelii	100	1	Actinomyces viscosus		
111 C	Strep. milleri	100	13	Spirillaceae Lbc acidophilus Strep. milleri	80 19 1	160	Spirillaceae Propionibact. acnes Strep. mutans Lbc acidophilus Strep. constellatus	32 28 22 13 5	117	Strep. milleri		
113 D										Propionibact. acnes		
115 B	Strep. mitior	100	7	Actinomyces naeslundii Anaerobic micrococci Strep. mitior Rothia dentocariosa	33 33 22 12	9	Actinomyces israelii Actinomyces viscosus Strep. mitior Veillonella parvula	41 35 12 12	17	Strep. mitior		

116 D	Lbc sp. Rothia sp.	98 147	Bifidobact. eriksonii Lbc acidophilus Lbc salivarius Veillonella alcalescens Strep. milleri Strep. sanguis	55 226	Bifidobact. eriksonii Strep. constellatus Lbc acidophilus Bacteriodes oralis Actinomyces viscosus Actinomyces odontolyticus	60 183	Strep. milleri Lbc acidophilus
119 D							
122 E					Bifidobact. eriksonii Propionibact. acnes	82 54	Gram positive rod*
124 E					Eubact. alactolyticum	202	Eubact. alactolyticum
125 C	Lbc sp.	100 72	Lbc fermentum Lbc casei var. rhamnosus Lbc acidophilus Veillonella alcalescens	100 48	Lbc fermentum	100 209	Lbc fermentum
					Lbc acidophilus Lbc buchneri Veillonella parvula Strep. mitior Lbc casei var. rhamnosus Lbc brevis	27 129	Strep. mitior Lbc casei Veillonella alcalescens
126 C							Lbc fermentum

* Lost.

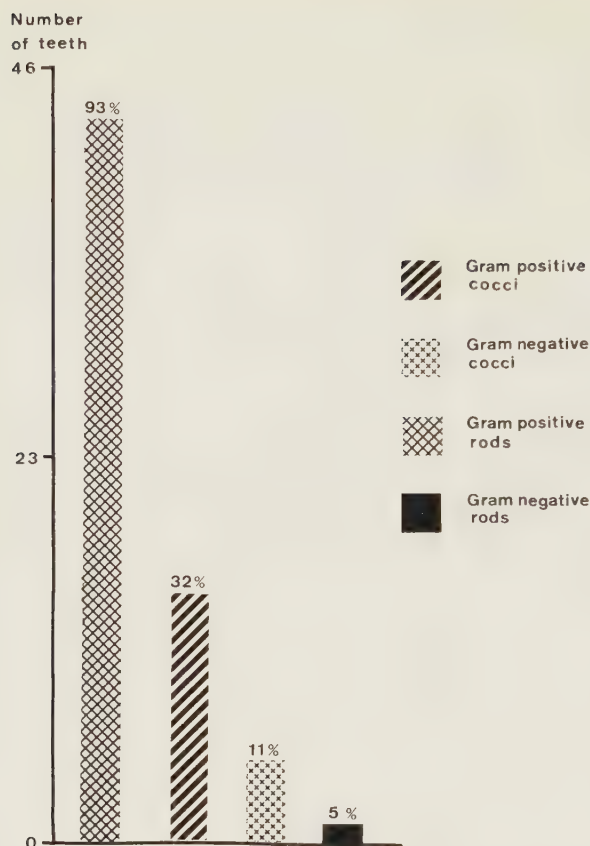


Fig. 8: 1. Number of positive isolations of Gram positive and negative cocci and rods.

and summarized in Tables 8: 2—5. It should be observed that in some cases the percentage figures in Table 8: 1 are based on a small number of CFU (colony forming units). Such examples are the results for teeth nos. 38 and 49.

Group I

Strains identified as *Actinomyces spp.*, *Arachnia spp.*, *Bifidobacterium spp.*, *Eubacterium spp.*, *Propionibacterium spp.*, and *Rothia spp.* were isolated from 29 of the teeth. The numbers of positive isolations for these genera are given in Table 8: 2. Each of the genera was demonstrated in less than 25 per cent of the teeth. The figures for the relative number of CFU on blood agar showed that *Arachnia spp.* and *Eubacte-*

Table 8: 2. Incidence of *Actinomyces* spp., *Arachnia* spp., *Bifidobacterium* spp., *Eubacterium* spp., *Propionibacterium* spp. and *Rothia* spp. Group I isolates.

	Isolated from number of teeth:		Observations at the primary isolation:			
		In per cent of analysed teeth	In number of teeth given the relative proportion of CFU for each genus/species on blood agar 7H and 2H have amounted to:			Growth only ob- served in TA-Ser medium
			≤ 49 %	50—99 %	100 %	
<i>Actinomyces</i>	5	11				
israelii	4		3		1	
naeslundii	3		3			
viscosus	3		2			1
odontolyticus	2		1	1		
sp.	2		1		1	
<i>Rothia</i>	2	4				
dentocariosa	1		1			
sp.	1		1			
<i>Bifidobacterium</i>	9	20				
eriksonii	5		3	2		
sp.	4		1	2		1
<i>Arachnia</i>	8	17				
sp.	8		1	2	2	3
<i>Propionibacterium</i>	9	20				
acnes	8		5	2		1
sp.	1		1			
<i>Propionibacteriaceae</i>	1	2			1	
<i>Eubacterium</i>	8	17				
alactolyticum	8		2	3	2	1

rium spp. often constituted more than 50 per cent of the counts, while corresponding figures for the other genera were smaller or varied more widely.

The demonstration of *Actinomyces spp.*, *Bifidobacterium spp.* and *Rothia spp.* are in line with several previous reports of bacteriological studies of the carious dentine (Howe and Hatch 1917, Burnett and Scherp 1951 a, b, Onisi and Nuckolls 1954, 1958, Roth 1957, Batty 1958, Scardovi *et al.* 1971, 1974, Jordan and Hammond 1972, Loesche and Syed 1973).

Arachnia, which has recently been described as a new genus (Pine and Georg 1969), as well as *Eubacterium* and *Propionibacterium*, have been observed in material from dental plaque and oral infections (Vincent and Reyners 1947, Theilade and Gilmour 1961, Rasmussen *et al.* 1966, Gerencser and Slack 1967, Holmberg and Forsum 1973, Bowden *et al.* 1975). Only little is known about the presence of *Arachnia spp.* and *Eubacterium spp.* in carious dentine. In a study of the bacterial flora of soft carious dentine, Loesche and Syed (1973) observed a group of Gram positive rods which they did not assign to any particular genus. They suggested that these organisms might be members of the *Arachnia* and *Eubacterium*. The present results thus support the conception of Loesche and Syed. As far as can be ascertained no report has been published on the presence of *Propionibacterium* in carious dentine.

Group II

Lactobacillus-like isolates were observed in 22 teeth. The numbers of positive isolations are given in Table 8:3. *Lactobacillus casei* var. *rhamnosus* and *Lactobacillus casei* var. *casei* were most frequently observed (14 teeth). Among the other species *Lactobacillus acidophilus* and *Lactobacillus fermentum* were often demonstrated (9 and 5, respectively). When appearing on blood agar and in mixed cultures, the variety of *Lactobacillus casei* dominated, while the other species constituted less than 50 per cent of the CFU in the mixed cultures.

In 1915, Kligler observed a relationship between dental caries and the presence of *Bacillus acidophilus* (Moro). *Bacillus acidophilus* has since been regarded synonymous with *Lactobacillus spp.* (Buchanan *et al.* 1966, Burnett and Scherp 1968 p. 352). Kligler's paper (1915) seems to have been the first to give a comprehensive description of the today well known correlation between the lactobacilli and the carious process. As presented above, those species within genus *Lactobacillus*, which have previously been observed in the human oral cavity (Rogosa *et al.* 1953, Grubb and Krasse 1953, Davis 1955) and in carious dentine (Camilleri and Bowen 1963, Shovlin and Gillis 1969, Loesche and Syed 1973), were demonstrated also in the present study.

Group III

Streptococcus-like isolates assigned to group III were observed in 9 of the teeth (Table 8:4). When they occurred in mixed cultures these isolates usually constituted only a minor part of the CFU (less than 50 %).

Table 8: 3. Incidence of *Lactobacillus* spp. Group II isolates.

	Isolated from number of teeth:		Observations at the primary isolation:			
		In per cent of analysed teeth	In number of teeth given the relative proportion of CFU for each genus/species on blood agar 7H and 2H have amounted to:			Growth only ob- served in TA-Ser medium
			≤ 49 %	50—99 %	100 %	
<i>Thermobacterium</i>	9	20				
<i>L. acidophilus</i>	9		7	1	1	
<i>L. salivarius</i> var. <i>salicinius</i>	1		1			
<i>Streptobacterium</i>	11	24				
<i>L. casei</i> var. <i>rhamnosus</i>	8		4	1	3	
<i>L. casei</i> var. <i>casei</i>	6		2	2	1	1
<i>L. plantarum</i>	2		1	1		
<i>Betabacterium</i>	11	24				
<i>L. fermentum</i>	5		1	1	1	2
<i>L. cellobiosus</i>	2				1 ¹	1
<i>L. brevis</i>	3		2	1		
<i>L. buchneri</i>	2		2			
<i>Lactobacillus</i> spp. ²	10	22				1

¹ Only organism growing on blood agar 2A.
² Isolated from blood agar incubated 2A or from TA-Ser medium.

Among oral species of the viridans streptococci, *Strep. sanguis*, *Strep. mutans*, *Strep. mitior* and *Strep. milleri* were demonstrated. *Strep. mutans* was first associated with carious dentine in the study of Clarke (1924). His observations have been verified by others (Maclean 1927, Onisi and Nuckolls 1958, Kreter and Dornauf 1970, Loesche and Syed 1973). Strains isolated from deep carious dentine and identified as *Strep. sanguis* seem to have been described first by Yardeni *et al.* (1959). This observation has later on been verified (Kreter and Dornauf 1970, Loesche and Syed 1973). In the present study, no isolate was observed which resembled *Strep. salivarius*. This is in agreement with previous reports. Krasse (1954) found this microorganisms in low numbers in plaque and Loesche and Syed (1973) rarely observed it in material from soft carious dentine.

The heterogenous group of streptococci, which does not produce demonstrable extracellular polysaccharides from sucrose, has previously been referred to as *Strep. mitis*. This species has recently been divided

Table 8: 4. Incidence of *Streptococcus* spp. Group III isolates and *Veillonella* spp., *Bacteroides* spp. and *Spirillaceae*. Group IV isolates.

	Isolated from number of teeth:		Observations at the primary isolation:			
		In per cent of analysed teeth	In number of teeth given the relative proportion of CFU for each genus/species on blood agar 7H and 2H have amounted to:			Growth only ob- served in TA-Ser medium
			≤ 49 %	50—99 %	100 %	
Group III-isolates						
<i>Streptococcus</i>	10	22				
S. sanguis	2		1		1 ¹	
S. mutans	4		2	1		1
S. milleri	3		3			
S. mitior	5		3	1		1
Group IV-isolates						
<i>Veillonella</i>	4	9				
V. alcalescens	3		3			
V. parvula	2		2			
<i>Bacteroides</i>	1	2				
B. oralis	1		1			
<i>Spirillaceae</i>	1	2	1			

¹ Only organism growing on blood agar 2A. Isolates resembling *Strep. sanguis* were not found on the other blood agar plates (7H and 2H) or in TA-Ser medium within the analysed portion of this tooth.

into two groups, *Strep. milleri* and *Strep. mitior* (Guthof 1956, Colman and Williams 1967, 1972). Both groups have been observed in the human oral cavity (Bowden and Hardie 1973 b, Mejáre and Edwards-son 1975). They were demonstrated also in the present study. Some strains of *Strep. milleri* produce hyaluronidase (Colman and Williams 1972). Thus, some of the isolates resembling *Strep. milleri* in the present study may be similar to those hyaluronidase-producing viridans streptococci, which have previously been thought to take part in the destruction of carious dentine (Parikh *et al.* 1965, Toto *et al.* 1968, Toto and Prendergast 1968).

Group IV

Gram negative cocci and rods were demonstrated in 6 teeth. They were found in mixed cultures and constituted less than 50 per cent of the CFU

(Table 8:4). Previous reports have indicated the presence of a high number of *Veillonella*-like organisms in plaque material collected from carious surfaces (Hemmens *et al.* 1941, Mazzarella and Shklair 1960, Loesche and Syed 1973). However, judging from the observations by Loesche and Syed (1973) and the present findings, these organisms seem to be less common in carious dentine.

Bibby and Hine (1938) found Gram negative bacilli and fusiforms in microscopic smears from carious dentine. On the other hand, these organisms were not observed by Loesche and Syed (1973) when they cultured soft carious dentine. In the present study Gram negative bacilli were observed in only 2 teeth. This suggests that Gram negative rods are rare in the deeper area of carious dentine.

Group V

This group consisted of anaerobic streptococci and micrococci, Gram positive anaerobic rods, *Staphylococcus spp.*, *Micrococcus spp.* and *Bacillus spp.* A number of heterogenous organisms which did not fit into any of the other identification groups were thus assigned to this group (Table 8:5). Members of the group were found in 17 teeth. The Gram positive anaerobic cocci were observed in 7 teeth. With the exception of the anaerobic *Streptococcus intermedius* (Holdeman and Moore 1974) and one isolate identified as anaerobic micrococci, the group V-isolates were all isolated from blood agar.

In all five isolates, which were lost before final identification, are also included in this group (Table 8:5).

Gram positive anaerobic cocci regarded as *Peptococcus* or *Peptostreptococcus* have previously been observed in dental plaque and gingival crevice (Gibbons *et al.* 1963, 1964, Socransky *et al.* 1963, Berg and Nord 1972). Less evidence is available about their presence in carious dentine. Davies' and Nemes' (1955) observation of small numbers of anaerobic Gram positive cocci in the deepest level of carious dentine and the present findings suggest, however, that the use of the sampling technique applied together with complete anaerobic conditions (Aranki *et al.* 1969, Holdeman and Moore 1972) might prove useful in the estimation of the incidence of these organisms in carious dentine.

Facultative anaerobic organisms belonging to *Micrococcaceae* have been observed in material from carious dentine (Burnett and Scherp 1951 a, b, Onisi and Nuckolls 1954, Yardeni *et al.* 1959, Shovlin and Gillis 1969) but, as a rule, in low incidence. These organisms were demonstrated in small numbers also in the present study.

Thus, judging from the qualitative representation of the micro-

Table 8: 5. *Incidence of anaerobic streptococci, anaerobic micrococci, Gram positive anaerobic rods, Staphylococcus spp., Micrococcus spp., Bacillus spp. and isolates lost before final examination.*

	Isolated from number of teeth:		Observations at the primary isolation:			
		In per cent of analysed teeth	In number of teeth given the relative proportion of CFU for each genus/species on blood agar 7H and 2H have amounted to:			Growth only ob- served in TA-Ser medium
			≤ 49 %	50—99 %	100 %	
<i>Anaerobic streptococci</i>	6	13				
<i>S. constellatus</i>	3		3			
<i>S. intermedius</i>	3			1		2
<i>Anaerobic micrococci</i>	2	4	1			1 ¹
<i>Gram positive anaerobic rods</i>	2	4	1	1		
<i>Micrococci</i>	3	7				
<i>Staphylococcus epidermidis</i>	3		1	2		
<i>Micrococcus saprophyticus</i>	1		1			
<i>Bacillus sp.</i>	1	2	1			
<i>Isolates lost before the final examination</i>	5	11	2		1	2

¹ Growth in mixed culture together with *Strep. mitior*.

organisms in group I—V, a variety of different genera and species were isolated from the deeper area of carious dentine.

Several groups of organisms, such as those of *Streptococcus*, *Lactobacillus* and *Actinomyces* have previously been observed. On the other hand, little or nothing seems to have been reported about the presence of such organisms as anaerobic streptococci, *Arachnia*, *Eubacterium* and *Propionibacterium* in deep carious dentine.

The results of the analysis of the quantitative representation also revealed that the Gram positive rods and filaments were predominant. In contrast with other groups of organisms, they often constituted more than 50 per cent of the CFU in the counts (Tables 8: 2—5). This was also observed when the mean percentage figures were calculated (Table 8: 6). On blood agar 7H both organisms with short and long generation time should have greater possibility to grow than on the other media. The most reliable observations should therefore be those made in these

plates. The proportion between different groups isolated from blood agar incubated 7H, appear to warrant the following conclusions. Gram positive bacteria constituted at least 95 per cent of the mean percentage counts and within this group the rods were predominant (81 %). The mean figure for the anaerobic bacteria was 51 per cent also with a preponderance of Gram positive rods.

The figures for the mean distributions of Gram positive anaerobic and facultative anaerobic cocci on the blood agar incubated 7 days anaerobically (7H), were roughly equal (Table 8:6). Judging from the mean figures of the anaerobic cocci and anaerobic rods these organisms seem to be more dependent on the culturing procedures. They gave higher mean figures on blood agar incubated (7H) than the facultative anaerobic isolates, which were more evenly distributed on the different media, and they are presumably less dependent on the culturing procedures.

The combinations of organisms in the different teeth revealed that *Actinomyces spp.* were observed in 6 teeth combined with *Streptococcus spp.* *Lactobacillus spp.* were demonstrated together with *Bifidobacterium spp.* and with *Streptococcus spp.* in 5 teeth, respectively. The number of teeth with any other combination of organisms were less than five. Considering the number of positive isolations for each genus and species, the results do not lend support to the assumption of microbial symbiosis.

Contaminations of cultures

Is it possible that some of the above-mentioned genera or groups of organisms might represent culture contaminants. This might be of importance to consider since in several of the cultures of dentine material on blood agar plates small numbers of CFU were found—sometimes only one or two CFU. If culture contaminants are present in such cases they may seriously interfere with the results. Judging from previous observations (Mc Dade *et al.* 1966, Johnson and Cummins 1972) and the findings in the present study (Chapter 2, pp. 43—45) isolates resembling facultative anaerobic *Micrococcaceae*, *Bacillus spp.* and *Propionibacterium spp.* might be contaminants.

Facultative anaerobic *Micrococcaceae* were demonstrated in small numbers. After improvement of the aseptic technique (part III of the study), these organisms were no longer observed (Chapter 2, pp. 23—24). This observation, and the fact that the normal habitat of several groups of organisms within *Micrococcaceae* is the human skin, suggest

Table 8: 6. *Mean percentage distribution.*¹

	Culturing procedures:					
	7H or T ²		2H ²		2A ²	
	mean	range	mean	range	mean	range
<i>Gram positive anaerobic rods</i>	44	0-100 ³	7	0-100		
Arachnia spp.	12					
Bifidobacterium spp.	9		2.5			
Eubacterium spp.	11		4.5			
Propionibacterium spp.	11					
Gram positive rods	1					
<i>Gram positive facultative anaerobic rods</i>	38	0-100	35	0-100	25	0-100
Actinomyces spp.	5		5			
Rothia spp.					< 1	
Lactobacillus spp.	33		30		25	
Bacillus spp.	< 1					
<i>Gram positive anaerobic cocci</i>	7	0-100	1	0-33		
streptococci	7					
micrococci			1			
<i>Gram positive facultative anaerobic cocci</i>	6	0-100	5	0-50	9	0-100
streptococci	6		4		8	
micrococci			1		1	
<i>Gram negative anaerobic cocci</i>	< 1	0-20	< 1	0-11		
Veillonella spp.	< 1					
<i>Gram negative anaerobic rods</i>	< 1	0-32	1.5	0-80		
Bacteroides spp.	< 1					
Spirillaceae	< 1					
<i>Isolates lost before final examination</i>						
Gram positive and negative cocci and rods	4	0-100				

Symbols

¹ The mean figures derived from the sum of the percentage figures of all the isolates presented in Table 8: 1 divided with the total number of analysed teeth (n = 46). *Note* the percentage of teeth in which each genus was observed is given in Table 8: 2—5.

² The relative proportion of CFU for the different groups and genera as observed after the incubation of dentine material; 7H or T: the result on blood agar incubated 7 days anaerobically (7H); when no growth was observed on the blood agar the organism growing in thio-glycollate-serium medium (T) was registrated with the percentage figure 100. 2H: the result on blood agar incubated 2—3 days anaerobically. 2A: the result on blood agar incubated 2—3 days aerobically.

³ 100 per cent means that the organism has been observed in pure culture.

that in the pathogenesis of dentine caries these bacteria may be involved to a less extent than the other organisms found.

Propionibacterium spp. have been considered a common anaerobic contaminant (Cato *et al.* 1970, Johnson and Cummins 1972). On the other hand, this organism has been found in human dental plaque (Rasmussen *et al.* 1966, Holmberg and Forsum 1973, Bowden *et al.* 1975). Its presence in the acid environment of carious dentine (Dirksen *et al.* 1963) is probable, since most of the *Propionibacterium*-like isolates were aciduric. However, the figures given in Table 8: 2 on the incidence of *Propionibacterium* in carious dentine, might be too high.

In the present study one isolate was regarded as *Bacillus sp.* This isolate was presumably a contaminant (Mc Dade *et al.* 1966, Chapter 2 pp. 32—36). It should, however, be observed that spore-forming bacteria resembling *Bacillus spp.* have previously been isolated from carious dentine more frequently than in the present study (Kligler 1915, Burnett and Scherp 1951 a, Onisi and Nuckolls 1954, 1958). Such variations in the frequency might be due to differences in the methods applied. But another explanation is also possible. Owing to differences in the dietary habits between different populations it is possible that the food can be more or less contaminated with soil. If present in appropriate amounts in food, spore-forming soil bacteria may occur within the human oral cavity and enter carious lesions. Such a conception is supported by the observations of Loesche *et al.* (1972). They considered that spore-forming soil bacteria may be more common in the oral cavity of individuals more exposed to soil contamination. It is possible that within the population from which the material for the present study was collected, the hygienic standard of the food was such that establishment of soil bacteria in the oral cavity is less likely.

The incidence of bacteria in relation to the sampling and cultivation techniques

Table 8: 1 shows that the number of CFU as well as the number of genera and species demonstrated in the different teeth varied widely. In some teeth the number of CFU was larger than in others. Many of these cases showed several genera and species including facultative anaerobic streptococci and Gram negative bacteria (for example teeth 68, 104 and 116). The samples were collected with a dental drill (Chapter 2, pp. 19—21). Because of the size of the drill (diameter approx. 1 mm) compared with the diameter of the dentine tubules which often are less than 2 μ m (Fromme and Riedel 1970), the grinding presumably included a large number of dentine tubules. Those tubules

situated in the area of the advancing bacterial front and involved in the samples might have contained a mixed flora. On the other hand, it is possible that each tubule could have contained a pure culture of only one species but that several tubules—each with a pure culture—may have been involved by the drill. This could be one possible reason why some cultures, consisting of several types of bacteria, were observed. But to a certain extent the findings in these teeth with a mixed flora also resemble the bacterial flora of “soft carious dentine” as reported by Loesche and Syed (1973). Thus, it can also be possible that in some teeth in the present study, the sample were not taken from the extreme depth of the bacterial front line, but from more destroyed parts of the lesion. It is, however, difficult to determine which of these two suggestions that might be the most reasonable explanation. On the other hand, in the teeth with a small number of CFU only few genera and species were generally demonstrated (for example teeth 38, 49, 79 and 88). Such cultures might better represent those, presumably few, bacteria penetrating deeply into the dentine. In these latter teeth Gram positive rods were often demonstrated.

The observation in the present work of predominance of Gram positive rods in the deeper area of carious dentine contrasts with several previous studies of the field (Clarke 1924, Maclean 1927, Burnett and Scherp 1951 a, b, Yardeni *et al.* 1959, Plathner and Binus 1965, Parikh *et al.* 1965, Toto *et al.* 1968, Kreter and Dornauf 1970 *inter alia*). In these studies the same part of carious dentine have been examined and observed to contain a larger incidence of Gram positive cocci, mainly streptococci. The variable results might be due to differences in the approach to the lesion when the samples were collected. Such a conception is supported by recent studies (McKay 1969, Sumney and Jordan 1973, 1974) where a sampling technique somewhat similar to that used in the present study was employed in order to analyse the bacterial flora of deep carious dentine. The results of those recent studies revealed that a large number of Gram positive rods might be present in the area. Furthermore, Onisi and Nuckolls (1954, 1958) also arrived at similar conclusions in a study where they clearly defined the area from which they collected their samples as “hard dentine with a definite colour change”.

The results of the above-mentioned studies (Onisi and Nuckolls 1954, 1958, McKay 1969, Sumney and Jordan 1974) and the observations in the present work, however, revealed great difference between the Gram positive rod-like flora. Onisi and Nuckolls (1958) isolated mainly organisms resembling *Actinomyces* and *Nocardia*. McKay (1969, 1971 personal communication) found exclusively *Lactobacillaceae*-like orga-

nims. Sumney and Jordan (1974) observed aerobic Gram positive diphtheroides with characteristics of genus *Arthrobacter*. In the present study lactobacilli and Gram positive pleomorphic rods and filaments were mainly observed but in a wide variety.

One possible explanation for differences observed in the bacterial flora has been presented by Sumney and Jordan (1974). They suggested that a unique bacterial flora in this deeper area exists for caries that originates from different surfaces of the dentition, such as from cemental or enamel surfaces. The conception could not be verified by the present study since almost all the teeth analysed had a lesion originating from an enamel surface. Within those few teeth (40 and 58) where the lesion originates from different surfaces of the dentition, such as from cemental or from enamel surfaces no special bacterial flora was found.

A second explanation might be that the caries activity in the dentition as a whole, or in the area of the mouth from which the tooth was extracted, could influence the condition in the deeper area of caries lesions. Fusayama *et al.* (1966), who studied the relationship between hardness, discoloration and microbial invasion in carious dentine observed an obvious difference in these parameters between, what they consider, acute and chronic caries. They found that the front line of softening and discoloration were far ahead of the bacterial front line in acute caries, while those three parameters were closer to each other in chronic caries. Their observation might indicate a more acidogenic and aciduric flora or a larger biochemical activity in acute caries lesions. In an attempt to reveal the consequence of caries activity in the present study, this parameter was estimated in the dentition as a whole and for each tooth separately at the collection. No differences were observed between the bacterial flora of those teeth taken from a dentition where the caries activity had been estimated as higher than that in teeth taken from a dentition with low caries activity. It should be pointed out, however, that caries activity might be difficult to estimate from a single observation (Quensel *et al.* 1952). On the other hand, Dirksen *et al.* (1962, 1963) observed that the pH of carious dentine was consistently lower at the base of the cavity than in more superficial parts of the lesion. Thus, a more constant acid environment might be present in the deeper area of carious dentine. This acid area might be less dependent on the fluctuation of pH in overlying dental plaque and on the caries activity in the dentition as a whole.

A third explanation might also be offered for the variability observed. It is based on the homogenization of the dentine material. In a separate study working with intact teeth, the size of dentine chips after homogenization was measured and found to vary from 1.8–163.8 μm

(see Chapter 2, pp. 32 and 45). Although the hardness of dentine at the bacterial front in carious lesions is lower than that of intact dentine (Fusayama *et al.* 1966) and thus carious dentine is easier to break down, pieces of dentine chips with different sizes might have been present in the inoculum. Within this chips various amounts of bacteria might have been situated in such a way that they could not be able to grow out on the media. The microbial variability observed which might be dependent on these circumstances could be illustrated by the results in some of the teeth (Table 8: 1). From tooth 47, for example, *Propionibacterium sp.* and *Arachnia sp.* were isolated from the blood agar incubated 7H, while *Eubacterium sp.* was isolated from the blood agar incubated 2H. In teeth 62, 96 and 110 for example similar variations between the cultures on blood agar 7H and 2H were observed. However, such variations were not a constant finding since culture of material from other teeth showed that the same species of organisms could be isolated from three of the media or from all four (44, 85, 86 and 115, for example). Furthermore, preliminary studies of some of the cultures following the first positive culture have indicated that the same organisms can sometimes be found in the first and in the following positive culture. Such examples are tooth 46, portion B and C, tooth 79, portions B and C, where only *Eubacterium sp.* was found in all four portions.

Thus, although the variability observed in the flora within the different teeth might be dependent to a certain extent on the sampling technique, there is presumably a real variability in the bacterial flora. Such a conception is supported by previous observations on the dynamics and variability of the bacterial flora within the same region and between various regions in the mouth and within other cavities of the human body (Wagg 1965, Loesche and Gibbons 1966, Carlsson 1968 b, Gibbons and van Houte 1973, Gorbach *et al.* 1973, Bowden *et al.* 1975) as well as in carious dentine (Loesche and Syed 1973, Sumney and Jordan 1973, 1974). The variability observed in the present study within the deeper parts of the carious dentine might therefore not be regarded as unique. But judging from the wide range of variation of almost all the genera and species demonstrated (Table 8: 6), it might be suggested that the closer to the bacterial front line we take our samples, the more selective and simple will the flora appear in our cultures. This flora might be composed of those specific organisms, for example, in one case lactobacilli, in another case eubacteria, and so on—which at a specific moment find the environment suitable for growth. As a “pioneer”-bacterium this organism may enter and produce micro-colonies along the dentine tubules.

The role of bacteria in the deeper parts of the dentine carious process

Burnett and Scherp (1951b) postulated that "if certain bacteria are found regularly in close proximity to the advancing front of carious dentine they should be seriously considered as etiologically significant". Even if Gram positive rods are preponderantly found in the area, it is not quite obvious that such a postulate could be applied to this group of organisms as a whole. The microbial variability of the Gram positive rod-like bacteria in the area had to be taken into account. These bacteria presumably include various groups of organisms with quite different biochemical properties. Only some of these properties might be of importance in the pathogenesis of dentine caries.

Further, it has not been made quite clear which properties might be of great importance to explain the spread of bacteria in the dentine. But microbial production of organic acids and various enzymes which can be found ahead of the bacterial front line (MacGregor *et al.* 1956, Fusayama *et al.* 1966, Barnett and Scherp 1968, Larmas 1972) presumably prepare the dentine for the microbial invasion (Engel 1950, Konetzka *et al.* 1956, Armstrong 1958, Macdonald 1962 p. 112, Larmas 1972). These biological substances might not only originate from the bacteria in the deeper part of carious dentine, but they are probably the result of the biochemical activities of all the bacteria in the whole carious lesion. Further, it cannot be excluded that low molecular weight foodstuffs such as mono- and di-saccharides can diffuse into the deeper area of carious dentine (Stephan 1945, Caldwell and Bibby 1958, Dirksen *et al.* 1962, 1963). The substances produced by the bacteria and the foodstuff diffusing into the lesion might be of importance for the spread of the carious process. This might be illustrated by removal of the main part of carious dentine but leaving the deepest area untouched followed by a sealing of the cavity. In such cases the bacteria could certainly survive for long periods, but they do not seem to multiply to any appreciable extent (Besic 1943, Schouboe and Macdonald 1962, Fisher 1966, 1969) and the carious process appears to progress either extremely slowly or not at all (Besic 1943, Fisher 1969).

Thus, it appears that the bacteria of the deeper part of carious dentine might multiply and gain access in the dentine tubules preceded and supported by the biochemical substances produced by the total flora of the carious dentine and by the foodstuff that might diffuse into the deeper part of carious dentine. The damage of the dentine which takes place in the immediate environment of the invading bacteria might, at least partly, be due to compression resulting from the bacterial multiplication in the tubules (Symons 1970) and the release of bacterial

enzymes such as protease (Burnett and Scherp 1951 a, 1953, Larmas 1972) and hyaluronidase (Toto and Prendergast 1968, Linder 1974) during the bacterial multiplication and degeneration. Thus, although Gram positive rods could be preponderantly present in deeper parts of carious dentine they cannot be considered specific carious-promoting organisms in this environment. A more reasonable view would be that they are one of several causative factors in the pathological process of dentine caries.

Conclusion

In an endeavour to obtain more information about the flora at the advancing bacterial front of carious dentine, successive samples of dentine material were removed from the pulp chamber in the direction towards the lesion. The first positive culture was considered to represent the flora of the advancing bacterial front. In line with recent reports (MaKay 1969, Sumney and Jordan 1973) a large number of *Lactobacillus*-like organisms were observed in these cultures. Members of this genus were observed in approximately half of the teeth. This was the largest incidence for any genera observed. The other main part of the flora consisted of Gram positive pleomorphic rods and filaments. They were demonstrated in less than 25 per cent of the teeth. The description "Gram positive pleomorphic rods" or "filaments" has previously been given to strains collected from the carious dentine. In the present study it was, however, possible to identify the majority of the isolates which appeared with a pleomorphic cell shape as members of accepted genera and species. The previous observation of *Actinomyces spp.*, *Bifidobacterium spp.* and *Rothia spp.* in the area was verified (Howe and Hatch 1917, Burnett and Scherp 1951 a, b, Onisi and Nuckolls 1954, 1958, Roth 1957, Batty 1958, Scardovi *et al.* 1971, 1974, Jordan and Hammond 1972, Loesche and Syed 1973).

Other groups of pleomorphic rod-like isolates were identified as *Arachnia spp.*, *Eubacterium spp.* and *Propionibacterium spp.* Little or nothing has previously been reported about the presence of these bacteria in carious dentine.

In contrast with several other studies, the incidence of Gram positive cocci was low. This holds also for the polysaccharide-producing species *Streptococcus sanguis* and *Streptococcus mutans* which are considered to possess properties making it possible for them to contribute to the development of dental plaque and to the initiation of caries lesion (for a review, see Scherp 1971).

Considering the obvious range of variation for all the genera and

species observed, it is not possible to make any simple statement concerning a specific caries promoting capacity of the bacteria observed in the deeper area of carious dentine. Based on the results given in certain previous reports on the bacteria and on the biochemical activities in carious dentine and the observations in the present study, it would appear more reasonable to assume that the microorganisms in deeper parts of carious dentine are one of several causal factors in the pathological process.

9. General summary

During the last decade considerable interest has been focused on those bacteria which are assumed to be involved in plaque formation and enamel caries. Less attention has been given to those specific micro-organisms, if any, which might be involved in the pathological process in the deeper areas of carious dentine. Endeavours were therefore made to isolate the cultivable bacterial flora of the advancing front of the carious dentine lesion and second to identify isolated bacteria and assess the proportions of different bacteriological genera and species.

The work was introduced by methodological studies on the sampling technique and on the problems of bacterial contamination. In the main study 46 extracted teeth with lesions extending into the dentine, were investigated within four hours of extraction of the teeth. The surfaces of the teeth were disinfected with an iodine-ether-colophonium solution. Using an aseptic technique, successive samples of dentine were removed from the pulpal wall towards the lesion. When soft necrotic lesions were reached no further material was sampled. Each portion of dentine was homogenized and inoculated into three blood agar plates which were incubated for 2—3 days or 7 days in 94 per cent H_2 and 6 per cent CO_2 and 2—3 days aerobically. Material was also inoculated into one test-tube containing thioglycollate medium supplemented with horse serum; that tube was incubated for up to 14 days.

It was assumed that clinically intact dentine beneath the lesion does not contain cultivable bacteria. The first portion of material to give a positive culture should therefore contain bacteria from the advancing front of the lesion. In these cultures all colony forming units (CFU) were counted and totally 220 representative CFU isolated. The isolates were preliminarily identified. Based on this identification the isolates were tentatively brought into five groups (Table 2: 9, p. 38).

Identification

Group I

The isolates in this group consisted of 64 pleomorphic Gram positive rods and filaments. They were studied with gas chromatographic analysis, cell wall analysis, gel diffusion analysis and biochemical tests. Fourteen isolates resembled *Actinomyces israelii*, *Actinomyces naeslundii*, *Actinomyces odontolyticus* or *Actinomyces viscosus* or were only regarded as *Actinomyces spp.* Two isolates were assigned to *Rothia spp.* Ten isolates shared characteristics with *Bifidobacterium eriksonii* and another 4 isolates were identified only as *Bifidobacterium spp.* Eleven isolates shared characteristics with *Arachnia*. Nine isolates were identified as *Propionibacterium acnes* and one was tentatively diagnosed as *Propionibacteriaceae*. Twelve isolates were regarded as *Eubacterium alactolyticum*.

Group II

The characteristics of 84 isolates which had preliminarily been regarded as *Lactobacillus spp.* were studied with gas chromatographic analysis and with biochemical tests. The lactic acid isomers and amino acid composition of the cell walls were determined in selected organisms. Forty isolates closely resembled *Lactobacillus casei* var. *casei* and *Lactobacillus casei* var. *rharnosus*. Twenty-two isolates could be assigned to *Lactobacillus acidophilus* and *Lactobacillus salivarius* var. *salicinius*. Another 21 isolates were regarded as members of *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Lactobacillus brevis* or *Lactobacillus buchneri*.

Another group of 12 isolates were studied in a number of selected biochemical tests and identified as *Lactobacillus spp.*

Group III

Twenty-eight isolates were preliminarily identified as *Streptococcus spp.* They were examined further with morphological and biochemical tests.

Nineteen isolates together with 38 reference strains were subjected to numerical analysis. Another 9 isolates were studied with a number of selected biochemical tests.

Of totally 28 isolates, 7 were identified as *Streptococcus mutans*, 9 as *Strep. milleri* (Guthof), 2 as *Strep. sanguis* and 10 as *Strep. mitior*.

Group IV

This group consisted of 10 Gram negative isolates. They were characterized by gas chromatographic analysis and biochemical tests with prereduced sterilized media (PRAS-media). Six coccus-like isolates were identified as *Veillonella alcalescens* and *Veillonella parvula*. One rod-like isolate was regarded as *Bacteroides oralis* and another 3 crescent-shaped rods were provisionally regarded as *Spirillaceae*.

Group V

Sixteen Gram positive isolates could not be referred to any of the above mentioned groups. Eleven of the isolates were anaerobic and studied by means of gas chromatographic analysis and in biochemical tests under complete anaerobiosis. Of the eleven anaerobic isolates, 3 were regarded as *Streptococcus constellatus* (Moore and Holdeman), another 3 as *Streptococcus intermedius* (Moore and Holdeman). Two coccus-like isolates were regarded as anaerobic micrococci while 3 rod-like isolates were described only as Gram positive anaerobic rods. Five isolates were facultative anaerobic and studied with morphological and biochemical tests. Three of these isolates were identified as *Staphylococcus epidermidis*, one as *Micrococcus saprophyticus* and one as *Bacillus sp.*

Incidence in teeth studied

The dominating organisms observed were Gram positive bacteria. Gram positive rods and filaments were isolated from 43 (93 %) of the teeth; Gram positive cocci from 14 (32 %) Gram negative cocci from 5 (11 %) and Gram negative rods from 2 (5 %) (Fig. 8: 1, p. 108).

The high incidence of Gram positive bacteria could presumably be explained largely by the environment created in the deeper area of carious dentine. Evidence could be presented that the culturing technique might be responsible to a less extent for the low incidence of at least such Gram negative bacteria as are regarded as moderate anaerobes i.e. *Veillonella*, *Bacteroides* and *Fusobacterium*.

None of the genera or species observed were demonstrated in all the teeth. *Lactobacillus spp.* were found in 22 (48 %) of the teeth, which was the largest incidence observed. All the other genera were found in less than 25 per cent of the teeth, e.g. *Streptococcus spp.* in 10 (22 %), anaerobic streptococci in 6 (13 %), *Actinomyces spp.* in 5 (11 %).

Bifidobacterium spp. in 9 (20 %), *Arachnia spp.* in 8 (17 %), *Propionibacterium spp.* in 8 (17 %), and *Eubacterium spp.* in 8 (17 %) (Tables 8: 2—5, pp. 109—114).

No evidence could be presented for a specific combination of organisms, which could lend support to an assumption of microbial symbiosis.

The Gram positive rods and filaments dominated not only in the number of positive isolations (Fig. 8: 1). In those teeth, where they were observed they were abundant often constituting more than 50 per cent of the colony forming units (CFU) in the counts on the blood agar plates (Tables 8: 2—5). The Gram positive facultative cocci mainly *Streptococcus spp.* often appeared in mixed cultures and the CFU of these organisms often constituted less than 50 per cent of the counts.

The mean percentage distribution of the bacteria observed in relation to all the teeth studied (Table 8: 6, p. 116) revealed that the Gram positive *anaerobic* rods and filaments mainly consisting of *Arachnia spp.*, *Bifidobacterium spp.*, *Eubacterium spp.* and *Propionibacterium spp.* were most numerous (44 %). Gram positive *facultative anaerobic* rods and filaments consisting of *Actinomyces spp.*, *Rothia spp.*, *Lactobacillus spp.* and *Bacillus spp.* also had a high mean incidence (38 %). Corresponding figures for the Gram positive *anaerobic* cocci (*Streptococcus spp.*, micrococci) and Gram positive *facultative anaerobic* cocci (*Streptococcus spp.*, *Staphylococcus spp.*, *Micrococcus spp.*) were 7 and 6 per cent respectively. Gram negative *anaerobic* cocci and rods were found in less than 1 per cent.

As in other studies on the bacterial flora of the deeper area of carious dentine recently performed, the incidence of *Lactobacillus*-like organisms was found to be high. Previous observations of *Actinomyces spp.*, *Bifidobacterium spp.* and *Rothia spp.* in the area were verified. Little or nothing seems to have been reported previously on the presence of *Arachnia spp.*, *Eubacterium spp.* and *Propionibacterium spp.* in carious dentine.

In contrast with several other studies, the incidence of Gram positive *facultative anaerobic* cocci observed was low. This concerns also the polysaccharide-producing species *Streptococcus sanguis* and *Streptococcus mutans*. Gram positive *anaerobic* cocci, mainly *anaerobic streptococci*, were observed in approximately the same incidence as the *facultative anaerobic* cocci. Little has previously been reported on the presence of Gram positive *anaerobic* cocci in carious dentine.

A wide range of variation was observed for the genera and species demonstrated. In view of previous observations on the dynamics and variability of the bacterial flora within the same region and between various regions in the mouth, both in the presence and absence of

pathological processes, the variability observed in the present study might not be unique.

However, judging on the results in previous studies of the bacterial flora of carious dentine and the present findings it might be suggested that the closer the sampling site is to the bacterial front line in the lesion, the more selective and simple will the flora presumably appear in our culture. This flora might be composed of those specific organisms, for example in one case lactobacilli, in another case eubacteria and so on—which at a specific moment find the environment suitable for growth. As a “pioneer” bacterium the organism may enter and produce micro-colonies along the dentine tubules. Their penetration into the dentine is presumably preceded and supported by biochemical substances produced by the total flora of the carious dentine and by foodstuff which might diffuse into the area.

Considering the above discussed factors, it is not possible to make any simple statement concerning a specific caries promoting capacity of the organisms observed in the deeper area of carious dentine. It would appear more reasonable to assume that the microorganisms in deeper parts of carious dentine are one of several causal factors in the pathological process.

10. Key to the nomenclature

In the present study the nomenclature has mainly followed that described in the publications mentioned below.

Gram positive facultative anaerobic cocci

Breed *et al.* (1957), Baird-Parker (1970, 1971), Colman and Williams (1972).

Gram positive anaerobic cocci

Breed *et al.* (1957), Rogosa (1971 b, c), Holdeman and Moore (1974).

Gram negative anaerobic cocci

Rogosa (1971 d).

Gram positive facultative anaerobic rods

Rogosa and Sharpe (1959), Cowan and Steel (1965), Gerencser and Slack (1969), Holdeman and Moore (1972), Moore and Holdeman (1973).

Gram positive anaerobic rods

Holdeman and Moore (1972), Moore and Holdeman (1973).

Gram negative anaerobic rods

Breed *et al.* (1957), Holdeman and Moore (1972).

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